Vol. XXVII, No. 2 1933

JUN 13 1933

THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

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LONDON:
CAMBRIDGE UNIVERSITY PRESS
Fetter Lane, E.C.4
also
CHICAGO: The University of Chicago Press
(Agent for the United States)
BOMBAY, CALCUTTA, MADRAS: Macmillan
TOKYO: Maruzen Company, Ltd.

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1933

Price Fifteen Shillings net

PRINTED IN GREAT BRITAIN

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XLV. THE DETERMINATION OF CHLORIDE IN BLOOD.

By JOAN HOWARD NORRIS and GUSTAV AMPT.

From the Pathology Department, University of Melbourne.

(*Received December 5th, 1932.*)

In connection with an investigation, which is being made in these laboratories, into the acid-base equilibrium in cases of essential hypertension, routine determinations of the chloride in bloods, plasmata and sera were required. These called for a method not encumbered with difficult manipulations in fragile or special apparatus but capable, under all conditions, of yielding data of the necessary trustworthiness.

Several papers have appeared recently, in which the authors have shown that the quantitative determination of chloride in such media by the methods in common use [*e.g.* Van Slyke, 1923–4; Wilson and Ball, 1928] is subject to hitherto unsuspected errors [compare Morris and Morris, 1930; Sunderman and Williams, 1931], apparently associated with the use of an acid incineration mixture for the destruction of organic matter. It was therefore decided to investigate the possibilities of alkaline incineration, since this procedure, whilst efficient in destroying organic matter, should eliminate the possibility of loss of chlorine in the process.

Experimental.

Sunderman and Williams find in their experiments with olive oil and gum acacia that considerable losses of chloride occur on drying, which are directly attributable to the presence of these organic substances. The following series of tests was made to examine the influence of such organic additions when alkaline treatment is adopted.

Materials used.

Sodium chloride, A.R., an approximately $N/20$ solution.
Olive oil and gum acacia, proved chloride-free.
Sodium hydroxide, sticks, proved chloride-free.
Silver nitrate, $N/50$ solution.
Potassium thiocyanate, $N/50$ solution.
Ferric alum, saturated solution.
Nickel crucibles for fusions.
Gooch crucibles and asbestos for filtrations.

Procedure.

2 cc. sodium chloride solution were placed in a nickel crucible, 2 g. sodium hydroxide added, the mixture dried completely at 100–110° in an air-oven (this usually took 6 hours) and then fused below visible red heat (400–450°). The fused cake was dissolved in water, the solution transferred to a beaker and neutralised

to litmus with concentrated nitric acid. During neutralisation carbon dioxide is liberated energetically and precautions must be taken to avoid loss through spraying. The possibility also of losses of chlorine through the local oxidation of chloride must be borne in mind; rapid mixing of the acid and effective cooling ensure freedom from these losses.

Exactly 10 cc. silver nitrate were added and the solution made definitely acid with nitric acid. The silver chloride was filtered and washed on a Gooch crucible and the filtrate, which should be brilliantly clear, titrated with potassium thiocyanate according to Volhard.

Composition of material tested	Mg. NaCl found
1. 2 cc. NaCl, directly precipitated (control)	6·03 5·98
2. 2 cc. NaCl, 2 g. NaOH, treated as outlined	5·98 6·03
3. 2 cc. NaCl, 2 g. NaOH, 0·4 g. olive oil	6·01 5·98 6·03
4. 2 cc. NaCl, 0·4 g. olive oil, dried out completely before addition of 2 g. NaOH	5·98 5·97 5·97 5·98
5. 2 cc. NaCl, 2 g. NaOH, 4 drops olive oil, 0·5 g. gum acacia	5·97 6·03
6. 2 cc. NaCl, 4 drops olive oil, 0·5 g. gum acacia, dried out completely before addition of 2 g. NaOH	6·02

It was not found practicable to keep the volume of the solution prior to titration below 50–70 cc. When the difficulty of detecting the colour change at the end-point in solutions of such dilution is considered, the concordance in the above figures leaves no room for improvement. The extreme variation in the back-titration with thiocyanate was 0·05 cc., *i.e.* about one drop.

From these results it is evident that, in the alkaline method suggested, addition of organic substances such as olive oil and gum acacia does not cause losses of chloride such as Sunderman and Williams experienced with similar materials when working under acid conditions.

We next experimented with blood, plasma and serum. The procedure was exactly as before except that 1 cc. of the medium was used instead of 2 cc. sodium chloride solution. The following are some typical results.

Experiment	NaCl found mg./cc.
Blood A. 1 cc. (defibrinated), 2 g. NaOH	5·41 5·41 5·48
Blood B. 1 cc. serum, 2 g. NaOH	5·79, 5·79, 5·79
Blood C. 1 cc. (oxalated), 2 g. NaOH	4·81 4·77
1 cc. (oxalated) dried completely before addition of 2 g. NaOH	4·86 4·86

In another set of experiments potassium hydroxide was employed but it was found that, although of guaranteed purity, an appreciable correction on account of chloride was necessary. No outstanding advantage attended its use.

	Experiment	NaCl found mg./cc.
Blood D.	1 cc. whole blood, 2 g. KOH	4·91 4·94
	1 cc. whole blood dried completely before addition of 2 g. KOH	4·95
	1 cc. whole blood, 2 cc. NaCl solution, 2 g. KOH	10·90 10·96
	1 cc. whole blood, 2 cc. NaCl solution, dried completely before addition of 2 g. KOH	10·89

These figures show not only that no real loss of chloride occurred when the blood was dried, but also that there was nothing present in the blood to cause any significant loss when the normal chloride content was increased by the addition of sodium chloride.

Our conclusions from these experiments are that

(i) the alkaline method is at least capable of giving concordant values;

(ii) the losses experienced by Sunderman and Williams did not occur during the drying out of the medium before proceeding with the analysis for chloride, but must have taken place in the operation of destroying the organic matter with concentrated nitric acid. Their figures showed such striking losses (up to 30 % of the total chloride) that we felt a desire to reproduce them and, if possible, to determine which step in their procedure was responsible for the discrepancies.

The apparatus used in these attempts consisted of a reaction flask of about 100 cc. capacity, connected by a ground glass joint with a Geissler absorption bulb. While the flask was being heated a current of chlorine-free air was aspirated through the train; this swept any volatile acidic products into the Geissler bulb where they were effectively absorbed in a solution of *N* NaOH. At the conclusion of the experiment, the alkaline absorbent was transferred to a beaker, acidified with nitric acid and examined quantitatively for chloride in the usual way.

A preliminary test in which 2 cc. sodium chloride solution were evaporated with concentrated nitric acid—the familiar aqua regia reaction during which chlorine and nitrosyl chloride are evolved—showed conclusively that, within the limits of experimental error inherent in this method, there was no nett loss of chlorine from the system; any chlorine volatilised from the reaction flask was absorbed in the Geissler bulb.

In another experiment the important observation was made that no detectable decomposition of silver chloride took place on heating it with concentrated nitric acid at 100° for several hours.

The inorganic phase, therefore, is not responsible for any losses.

The effect of adding olive oil and gum acacia was next examined. 2 cc. *N*/20 sodium chloride solution were introduced into the reaction vessel, followed by 0·2–1·0 cc. of an aqueous emulsion containing 15 % olive oil and 10 % gum acacia. In one series of experiments these reagents were dried completely at 100–110° before further treatment. 10 cc. silver nitrate and 5 cc. concentrated nitric acid were then added, the flask immersed in a boiling water-bath and chlorine-free air aspirated through the apparatus. Digestion was carried on for periods varying from 4 to 8 hours. In some cases potassium permanganate was added also, as recommended by Van Slyke. The acid liquid remaining in the flask, and the sodium hydroxide solution in the Geissler bulb were then examined quantitatively for chloride.

A typical example of such a series is the following:

NaCl found by direct titration of 2 cc. sodium chloride solution, mg.	5·80, 5·76
NaCl found after tests described:	
(*a*) materials not dried out	5·76 (in Geissler—nil)
(*b*) materials dried completely	5·76 (in Geissler—nil)

Of special interest, because it stands at variance with the experiences of Sunderman and Williams, is the fact that the whole of the chloride in the reaction mixture was retained in the flask. This, after all, should have been anticipated since

(i) there is no chemical reason why organic materials of the nature of olive oil should, by simple evaporation with a neutral chloride solution, yield volatile organic chlorides by esterification;

(ii) the chlorine of silver chloride formed in the reaction vessel remains anchored throughout the test.

In another series of experiments we determined the chloride content of a blood by both the alkaline fusion and the Van Slyke methods. The results of our final group of tests showed complete agreement:

		NaCl found mg./cc.
Blood E.	(*a*) Alkaline fusion method	7·02 6·99
	(*b*) Van Slyke method	7·02 (in Geissler—nil)

Such was not always the case. Not infrequently an erratic value was obtained by the acid method which was always low, but never as much as 10 % low.

Sunderman and Williams are very definite in their statement that losses of chloride occur under certain conditions in the presence of organic matter. We have been unable to confirm this and, moreover, our work does not enable us to offer any suggestion which might help to explain the systematic losses they observed.

There is a feeling of security with the alkaline method that the results are readily reproducible. In our hands the Van Slyke method has worked reasonably well on the whole, but there has been the occasional erratic result without an obvious explanation; this naturally attaches an element of uncertainty to any single determination.

Conclusion.

The advantages claimed for the alkaline fusion method of destroying organic matter before proceeding with the determination of chlorides, set out briefly, are:

(1) a simple technique in robust apparatus,

(2) invariable reproducibility of results,

(3) end-point sensitiveness of one drop $N/50$ thiocyanate solution (equivalent to 0·06 mg. NaCl),

(4) uniform concordance within the limit stated.

We desire to acknowledge the interest shown by Professors P. MacCallum and E. J. Hartung in the progress of this work, and also the many facilities extended by them in the Pathological and Chemical Laboratories of the University of Melbourne.

REFERENCES.

Morris and Morris (1930). *Biochem. J.* **24**, 1716.
Sunderman and Williams (1931). *J. Biol. Chem.* **92**, 99.
Van Slyke (1923–4). *J. Biol. Chem.* **58**, 523.
Wilson and Ball (1928). *J. Biol. Chem.* **78**, *Proc.* l, 40.

XLVI. THE EFFECT OF HALOGEN SALTS ON TRYPTIC DIGESTION.

By WINIFRED MARY CLIFFORD.

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(*Received January 28th, 1933.*)

The effect of halogen salts on salivary and peptic digestion has been described previously [Clifford, 1925; 1927; 1928], and in this paper an account is given of their action on tryptic digestion.

The earliest work on the subject appears to be that of Pfeiffer [1884] who found that, with the exception of sodium carbonate, all salts, especially sodium chloride, retard tryptic digestion. Later, Weiss [1904] found that sodium chloride in strong concentration retarded tryptic action whilst in weak concentration it accelerated it. With sodium bromide and iodide he obtained similar though less marked results, and the potassium salts acted in the same way as the sodium salts. Unfortunately, his experiments were carried out using solutions of equal percentages and not of equal molecular concentrations, and this makes the results difficult to compare.

Robertson [1906] found that all salts accelerated, but stated that chlorides, nitrates, and sulphates accelerated tryptic action least, as compared with cyanides and salts of fatty acids.

In the experiments to be described an attempt was made to investigate the action of equimolecular solutions of halogen salts on the tryptic digestion of protein.

Experimental methods.

The substrate used was dried ground fish protein prepared as follows.

Cod muscle, freed from skin and connective tissue, was passed through a fine mincer. It was then covered with three times its volume of 85 % alcohol, shaken well and allowed to stand for a week. During this period it was shaken at intervals. The protein was wrung as dry as possible through cotton winceyette, placed in a shallow dish covered with muslin and left in the air near a radiator for about 48 hours. It was then quite dry and could be ground into a fine powder which keeps indefinitely and gives a large surface for digestion. Unlike blood-fibrin it does not form lumps but gives a homogeneous product.

4 g. of this dried fish protein were weighed into a 500 cc. flask and were covered with 200 cc. of 0·4 % sodium carbonate solution previously warmed to 38°. The flask was placed in a water-bath at 38° for 30 minutes, when 10 cc. of 6 % pancreas substance, also at that temperature, were pipetted into the mixture.

25 cc. were removed at once, and about 100 cc. of boiling water were added to stop digestion. A Sørensen determination was carried out on the 25 cc. by adding 30 cc. of formalin (1 part formalin, 2 parts water), a few drops of phenolphthalein, and then titrating against 0·1 *N* H_2SO_4. Similar titrations

were carried out at intervals, using 0·1 *N* NaOH when the digestion mixture became acid to phenolphthalein.

For experiments where the effect of halogen salts was to be noted, the appropriate weight of salt was dissolved in the 200 cc. of 0·4 % Na_2CO_3 before adding it to the weighed protein.

A typical experimental result is as follows:

4 g. dried fish protein; 200 cc. 0·4 % Na_2CO_3 with 0·125 *M* NaI; 10 cc. 6 % pancreas substance; 25 cc. taken out at intervals, treated with formalin and titrated.

Minutes	cc. of 0·1 *N* acid or alkali	cc. of 0·1 *N* acid produced by tryptic hydrolysis
0	6·0 cc. 0·1 *N* H_2SO_4	0·0
30	4·6 ,,	1·4
60	3·5 ,,	2·5
90	1·3 ,,	4·7
180	3·7 cc. 0·1 *N* NaOH	9·7
210	5·6 ,,	11·6
270	8·2 ,,	14·2
300	10·9 ,,	16·9

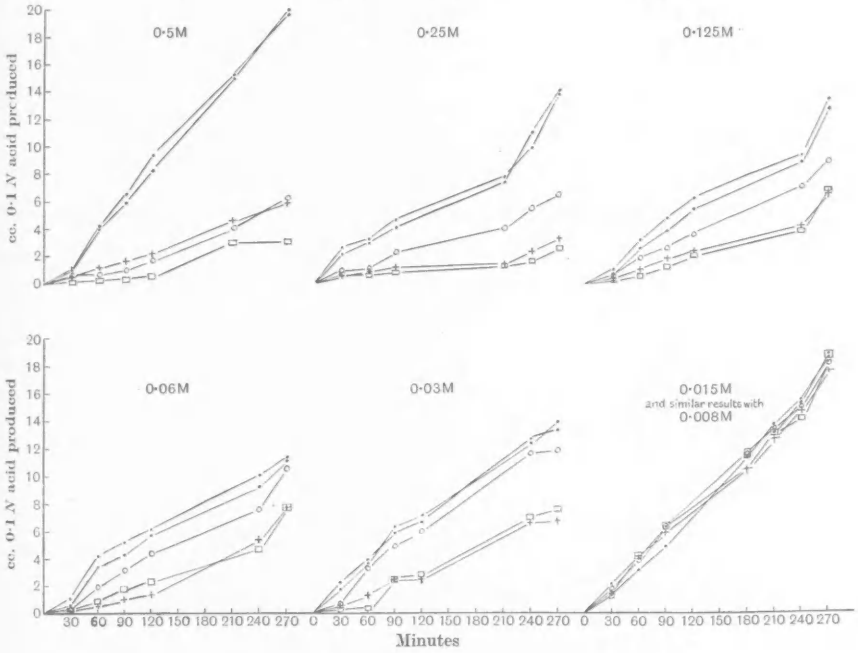

Fig. 1. Fluorides.

•—• Standard ×—× Lithium ○—○ Sodium +—+ Potassium □—□ Ammonium

In any set of experiments, two control flasks were used and one of each of the halogen mixtures of the concentration to be investigated.

Four separate batches of pancreas substance were used in the experiments

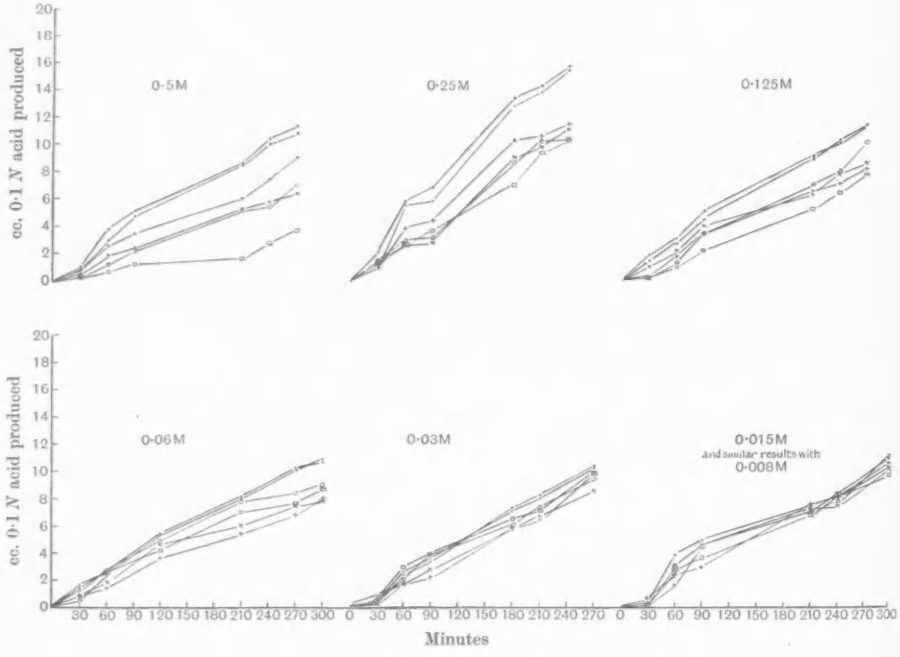

Fig. 2. Chlorides.

•—• Standard ×—× Lithium ○—○ Sodium +—+ Potassium □—□ Ammonium

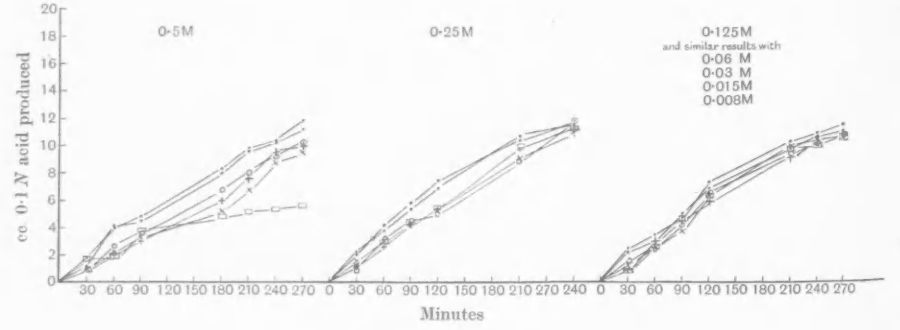

Fig. 3. Bromides.

•—• Standard ×—× Lithium ○—○ Sodium +—+ Potassium □—□ Ammonium

to be described, and at least three separate samples of each halogen salt. These various specimens all gave similar results. The concentrations of salt investigated varied from 0·5 *M* to 0·008 *M*.

Results.

Fluorides. Typical results with fluorides are shown in Fig. 1. From these curves it can be seen that sodium, potassium and ammonium fluorides in concentrations of 0·5–0·06 *M* are strongly inhibitory to tryptic digestion, but do

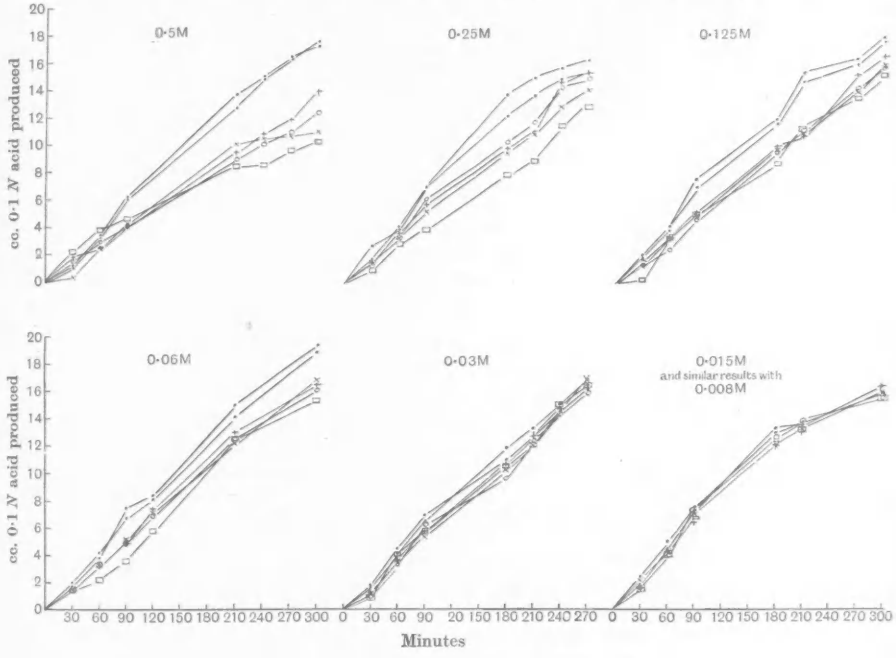

Fig. 4. Iodides.

•—• Standard ×—× Lithium ○—○ Sodium +—+ Potassium □—□ Ammonium

not actually stop it. This inhibition is greatest with ammonium and potassium fluorides, the sodium salt showing much less effect from 0·25 to 0·06 *M* and having hardly any action at 0·03 *M*. At concentrations of 0·015–0·008 *M* none of the three fluorides appeared to affect the rate of tryptic hydrolysis.

Chlorides. Graphs of experimental results are given in Fig. 2.

The chlorides, like the fluorides, have an inhibitory effect on tryptic digestion when used in the higher concentrations.

At 0·5 *M* this is most marked with the ammonium salt, but it is quite definitely seen with lithium, sodium and potassium chlorides.

At 0·25 *M* all four chlorides show the same depressant action, and this continues down to 0·06 *M*. With concentrations of 0·03–0·008 *M* there is little

if any effect on the rate of tryptic digestion. No accelerating action has ever been seen with any concentration of chloride.

Bromides. Representative graphs are shown in Fig. 3. The four bromides used showed the least effect of all the halogens. At a concentration of 0·5 *M* there was a slight depression with the lithium, sodium and potassium salts and a rather more marked one with ammonium bromide. At 0·25 *M* the inhibitory action was hardly noticeable, and in concentrations from 0·125 to 0·008 *M* there was no effect of any kind.

Iodides. Curves of typical results are shown in Fig. 4. From these it can be seen that there is a definite depressing action in concentrations of 0·5–0·25 *M* and a slight inhibition in concentrations of 0·125–0·06 *M*. With concentrations of 0·03–0·008 *M* there is no effect on the rate of tryptic hydrolysis.

Discussion.

From these results it appears that the halogen salts of lithium, sodium, potassium and ammonium in concentrations of 0·5 *M* depress the rate of the proteolytic action of trypsin, though in different degrees. Fluorides and chlorides exert the greatest depressant action, bromides the least, whilst iodides slow the rate a little more than do the bromides, but much less than the chlorides.

With low concentrations of halogen salts the rate of tryptic hydrolysis was unchanged, but in no case was it ever hastened. The concentration at which the salts ceased to affect trypsin varied, being 0·015 *M* for fluorides, 0·03 *M* for chlorides and iodides and 0·125 *M* for bromides.

In every case with the higher concentrations the ammonium salt exerted the greatest depressing effect. This is probably due to the acid reaction of the ammonium halide solutions. It is remarkable however that in many cases, *e.g.* with 0·25 *M* NH_4Cl, although the digestion mixture was acid to phenolphthalein even before the addition of formalin, tryptic digestion proceeded as well as in mixtures containing 0·25 *M* NaCl, KCl or LiCl, which were strongly alkaline. Again in the bromide experiments the ammonium solutions were always more acid than those of the sodium, potassium and lithium salts, yet except in the highest concentration (0·5 *M*) this increase in acidity caused no slowing of tryptic activity. The alterations in rate of hydrolysis cannot therefore be attributed solely to changes of p_H in the digestion mixture.

With fluorides the relatively slight action of sodium fluoride as compared with the lithium and potassium salts is remarkable between 0·25 and 0·03 *M*. Above this concentration it is equally inhibitory, and below, all the fluorides are without effect. This is not due to a less acid reaction as the p_H values of digestion mixtures with potassium and sodium fluorides were identical.

Summary.

1. The digestion of fish protein by trypsin is delayed by the addition of the halogen salts of Li, Na, K and NH_4.

2. This depression is greatest with fluorides and chlorides, less with iodides, and least with bromides.

3. The depression is greatest with high concentrations of salt (0·5 *M*) and disappears at low concentrations.

4. In no concentration investigated (0·5–0·008 *M*) was there any accelerating action.

5. In the more concentrated solutions the ammonium salts exerted the most powerful depressant action.

6. Sodium fluoride was less strongly inhibitory than potassium or ammonium fluoride.

The expenses of this research were defrayed by a grant from the Medical Research Council.

REFERENCES.

Clifford (1925). *Biochem. J.* **19**, 218.
—— (1927). *Biochem. J.* **21**, 544.
—— (1928). *Biochem. J.* **22**, 1128.
Pfeiffer (1884). *Jahresber. Thierchem.* 278.
Robertson (1906). *Biochem. J.* **2**, 317.
Weiss (1904). *Z. physiol. Chem.* **40**, 480.

XLVII. THE DETERMINATION OF CALCIUM IN BLOOD-SERUM AND CEREBROSPINAL FLUID.

By ARTHUR HAROLD TINGEY.

From the Central Pathological Laboratory of the London County Mental Hospitals.

(*Received February 2nd, 1933.*)

The method for the estimation of calcium in general use is that of Kramer and Tisdall [1921] as modified by Clark and Collip [1925], depending on the direct precipitation of the serum-calcium as oxalate and titration of the latter with potassium permanganate.

Although this method has the advantage of simplicity the estimation cannot be made on fatty or opaque sera. This, coupled with the fact that in all specimens the calcium oxalate precipitates char on ignition, suggests that variations in the $KMnO_4$ titration may be partially due to the amount of organic matter adsorbed by the precipitate. Hirth and Klotz [1923] compared three sera by $KMnO_4$ and acidimetric methods, obtaining an average difference of 0·6 mg./100 cc., which they attributed to the titration by the $KMnO_4$ of organic matter. Later, Stanford and Wheatley [1925], by a similar comparison, obtained values differing by 4 %, which they attributed to personal error, their absolute recoveries by the acidimetric method being 3–7 % lower than by the $KMnO_4$ method.

There is, moreover, some doubt as to whether all the non-diffusible calcium, found by von Meysenbug *et al.* [1921] to form 30–40 % of the total serum-calcium, is precipitated; Van Slyke and Sendroy [1929] obtained 5–15 % more calcium when precipitated from deproteinised serum than when precipitated directly.

It is also questionable whether, in view of these difficulties, the clinical significance frequently attached to minor variations in serum-calcium, of the order of 0·3 mg./100 cc. as estimated by the Clark and Collip method, is justified.

In the endeavour to devise a procedure that will avoid these difficulties and give figures more approaching absolute values, the following methods have been investigated and compared.

(1) The Clark and Collip procedure and the incineration method of Trevan and Bainbridge [1926], *i.e.* the direct $KMnO_4$ titration as compared with the acidimetric titration of the ignited calcium oxalate precipitate.

(2) The Clark and Collip procedure and the method of Halverson and Bergeim [1917] as modified by Van Slyke, in the latter case deproteinising with acid of such strength that all the calcium is brought into solution and subsequently precipitating the calcium as oxalate and titrating with $KMnO_4$.

(3) The techniques for purification by washing of the calcium oxalate precipitate of Clark and Collip [1925], Halverson and Bergeim [1917] (as modified by Van Slyke and Sendroy [1929]), and Stanford and Wheatley [1925], since it is to the washing technique that the maximum error (± 3 %) of the Clark and Collip method is usually attributed. Clark and Collip require one washing with 2 % NH_4OH with draining by inversion of the tube; Halverson and Bergeim

require two washings with 2 % NH_4OH, withdrawing the supernatant fluid by suction; Stanford and Wheatley require two washings with saturated calcium oxalate solution.

(1) *Comparison of the Clark and Collip and Trevan and Bainbridge methods.*

For the Trevan and Bainbridge estimations, the precipitates were dissolved in $N/100$ H_3PO_4 (leaving overnight) and the titration made against $N/100$ NaOH, using bromophenol blue as the indicator and taking as the end-point a colour between buffer solutions p_H 4·0 and 4·2. It was found that, after gradual heating of the oxalate precipitates in the oven at 160° for an hour, subsequent heating over the direct Bunsen flame for 20–30 seconds gave the highest recovery from $CaCl_2$ solution and the least difference between the two methods when applied to serum.

Estimations were made mostly on pooled sera and C.S.F. and at least two estimations were made on each sample. For each method, most of the duplicates agreed to ± 1 % for the C.S.F. specimens; and, for sera, about one-half agreed to ± 1 % and the remainder to ± 2 %.

Forty-eight samples of serum were compared, and in every case the Clark and Collip method gave the higher figure; the average values being 10·4 mg./100 cc. (C. and C.) and 9·7 mg./100 cc. (T. and B.), with an average difference of 0·7 mg./100 cc. (highest 1·7, lowest 0·2).

Fourteen samples of C.S.F. gave average values of 5·7 mg./100 cc. (C. and C.) and 5·4 mg./100 cc. (T. and B.), with an average difference of 0·3 mg./100 cc. (highest 1·0, lowest 0·0).

Of the calcium added to 5 samples of serum and C.S.F. in the form of a 10 mg./100 cc. $CaCl_2$ solution; 106 and 101 % were the respective average C. and C. recoveries; and 98 % the average T. and B. recovery from both serum and C.S.F.

The absolute recoveries from $CaCl_2$ solutions were 100 % by C. and C. (average 9 estimations) and 98 % by T. and B. (average 11 estimations).

Table I.

		mg. Ca/100 cc.			
Serum	C. and C.	10·0	10·2	10·3	10·4
	T. and B.	9·1	9·6	9·2	10·0
C.S.F.	C. and C.	5·0	5·8	5·6	5·7
	T. and B.	4·9	5·6	5·4	5·4

Table II.

	Serum	0·1 mg. Ca added to 2 cc. sample	Recovery %
	mg. Ca/100 cc.		
C. and C.	10·1	15·4	106
,,	10·4	15·7	106
,,	9·5	14·7	104
,,	10·7	16·1	108
T. and B.	10·0	14·6	92
,,	9·9	14·9	100
,,	9·7	14·9	104
,,	10·1	15·1	100

Table III.

	C.S.F.	0·1 mg. Ca added to 2 cc. sample	Recovery %
	mg. Ca/100 cc.		
C. and C.	5·8	11·0	104
,,	5·6	10·6	100
,,	5·7	10·7	100
,,	5·5	10·6	102
T. and B.	5·6	10·4	96
,,	5·4	10·4	100
,,	5·5	10·5	100
,,	5·0	10·0	100

Hendriks [1929], in comparing the C. and C. and T. and B. methods on sera, obtained (as an average of 8 estimations) values of 10·7 mg./100 cc. and 10·5 mg./100 cc. respectively; but, in this investigation, the differences were higher throughout.

The results are further exemplified in Tables I, II and III, which represent absolute comparisons on four specimens of serum and C.S.F. respectively.

It will be observed that the $KMnO_4$ titration gives a higher figure than the acidimetric titration following ignition of the oxalate precipitate.

(2) *Comparison of the Clark and Collip and Halverson and Bergeim methods.*

The method of Halverson and Bergeim was carried out in the manner described by Peters and Van Slyke [1932]. The washing technique adopted was that of Clark and Collip in each case.

Twelve samples of serum were compared, and in most cases the H. and B. method gave the higher figure; the average values being 11·4 mg./100 cc. (H. and B.) and 11·1 mg./100 cc. (C. and C.), with an average difference between the two methods of 0·3 mg./100 cc. (highest 1·3, lowest, −1·1). Three extremely fatty sera estimated by the H. and B. method gave normal values.

Six samples of C.S.F. gave the same average value of 5·7 mg./100 cc. by each process, with an average difference of 0·0 (highest 0·3, lowest − 0·3).

Of the calcium added to 4 samples of serum and C.S.F. in the form of a 10 mg./100 cc. $CaCl_2$ solution, the average H. and B. recoveries were 99 % from serum and 100 % from C.S.F.

The H. and B. method gave an average absolute recovery from $CaCl_2$ solution of 102 % (average 4 estimations).

$KMnO_4$ and incineration methods, carried out in duplicate after deproteinisation on 5 cc. serum and C.S.F., gave similar values (serum, 11·1 H. and B., 11·2 T. and B.; C.S.F., 6·6 H. and B., 6·7 T. and B.). This is in agreement with the work of Heiduschka and Schmidt-Hebbel [1932].

These results are further exemplified in Tables IV, V and VI, which represent absolute comparisons on four specimens of serum and C.S.F. respectively.

Table IV.

		mg. Ca/100 cc.			
Serum	C. and C.	11·3	11·9	11·0	11·2
	H. and B.	11·6	11·8	11·6	11·8
C.S.F.	C. and C.	5·4	6·0	5·5	5·6
	H. and B.	5·7	5·7	5·6	5·6

Table V.

	Serum	0·1 mg. Ca added to 2 cc. sample	Recovery %
	mg. Ca/100 cc.		
H. and B.	10·9	15·7	96
,,	11·1	16·1	100
,,	9·1	14·7	96
,,	12·1	17·4	106

Table VI.

	C.S.F.	0·1 mg. Ca added to 2 cc. sample	Recovery %
	mg. Ca/100 cc.		
H. and B.	5·6	10·7	102
,,	5·3	10·3	100
,,	5·5	10·5	100
,,	5·6	10·6	100

It will be observed that, in most instances, the H. and B. method gives the higher value. The relative significance in respect to the suggested organic matter attached to the C. and C. oxalate precipitate, and the increase of calcium concentration due to separation of combined calcium by acid deproteinisation, will be discussed later.

(3) *Washing methods.*

The results of the three methods carried out on calcium oxalate precipitated without deproteinisation and after deproteinisation are set out in Tables VII and VIII.

Table VII.

Serum-Ca in mg./100 cc.

	Serum No.	1	2	3	4	5	6	7	8	9	10	11	12	13	Av.
Without deproteinising	C. & C.	11·0	10·9	—	—	—	—	—	11·2	10·7	—	—	—	—	11·0
	H. & B.	10·8	10·6	—	—	—	—	—	11·0	10·8	—	—	—	—	10·8
After deproteinising	C. & C.	—	—	11·0	10·7	10·4	11·0	—	—	—	—	—	—	—	10·5
	H. & B.	—	—	11·6	10·8	10·4	11·0	—	—	—	—	—	—	—	11·0
Without deproteinising	H. & B.	—	—	—	—	—	—	10·9	11·0	10·8	—	10·8	—	—	10·9
	S. &. W.	—	—	—	—	—	—	11·2	12·0	11·9	—	12·0	—	—	11·8
After deproteinising	H. & B.	—	—	—	11·0	—	11·0	—	—	—	—	—	10·5	10·5	10·7
	S. &. W.	—	—	—	11·8	—	11·8	—	—	—	—	—	10·7	10·5	11·0

Table VIII.

C.S.F.-Ca in mg./100 cc.

	C.S.F. No.	1	2	3	4	Average
Without deproteinising	C. & C.	—	—	5·5	5·6	5·5
	H. & B.	—	—	5·5	5·6	5·5
After deproteinising	C. & C.	5·7	—	5·6	—	5·6
	H. & B.	5·8	—	5·7	—	5·7
Without deproteinising	H. & B.	—	5·7	5·5	—	5·6
	S. & W.	—	6·0	6·0	—	6·0
After deproteinising	H. & B.	5·8	—	5·7	—	5·7
	S. & W.	6·8	—	5·4	—	6·1

As will be seen from the Tables, in the case of serum, after deproteinisation the H. and B. technique gave higher figures than that of C. and C., and without deproteinisation, slightly lower results. In the case of C.S.F. there was no difference between the C. and C. and H. and B. techniques.

The S. and W. technique gave higher values than that of H. and B.; the difference being less after deproteinisation than without deproteinisation.

Discussion.

(1) The Trevan and Bainbridge method was found to be more liable to personal error than the Clark and Collip method because it was not always easy to adjust the time of incineration so that the minimum quantity of CaO was formed or so that no fusion with the glass of the test-tube occurred. The absolute recoveries, and the agreement of $KMnO_4$ and incineration values after deproteinisation, however, suggest that personal error did not account significantly for the lower T. and B. results. Further, although a micro- and not a micrometer burette was employed, the agreement of duplicates would indicate that this factor also did not account significantly for the lower results.

It seems probable that the discrepancy between the two methods was caused firstly by the titration by the $KMnO_4$ of organic matter, which might augment the true value by about 0·3 mg./100 cc. (corresponding to one drop of $N/100$ $KMnO_4$), and secondly by the combination of protein-sulphur and -phosphorus with the calcium during the incineration, which might diminish the true value also by about 0·3 mg./100 cc. (corresponding to one drop of $N/100$ NaOH).

A comparison of the results supports this explanation. The difference between the results obtained by each method was less for C.S.F. than for serum, which contains more protein than C.S.F.; and the accuracy obtained in the agreement of duplicates was greater for C.S.F. Further, the recoveries of added calcium also appear to relate to the protein content. The average recovery from serum of 106 % by the C. and C. method may be explained by the greater adsorptive surface provided by the added calcium; and for C.S.F., the recoveries of 101 % (C. and C.) and 98 % (T. and B.) were respectively greater and less only by the small amounts corresponding to the small protein content.

(2) The average of 3 % more calcium obtained by the Halverson and Bergeim method as a result of deproteinising, although not within the 5–15 % range of Van Slyke and Sendroy, may suffice to justify the method in preference to that of Clark and Collip. A further advantage is that fatty and opaque sera can be successfully analysed. The removal of the protein also gives greater relative accuracy to the titration.

The C.S.F. protein-calcium was insufficient to augment the average H. and B. value; although, in one instance, an extra 3 % was obtained.

99 % of added calcium was recovered by the H. and B. method from serum, and 100 % from C.S.F., there being, owing to the deproteinisation, no organic matter to be adsorbed.

(3) It is evident from the results on the washing technique that the method of Halverson and Bergeim is to be preferred, because this tends when compared with the C. and C. technique to lower the results obtained from non-deproteinised serum (probably owing to the solubility of titratable protein in the extra washing fluid) and to raise those obtained from deproteinised serum (there being in this case a finer precipitate, which tends to drain away upon inversion of the test-tube during the C. and C. washing).

Although the saturated calcium oxalate of Stanford and Wheatley fulfilled the author's requirements as to $KMnO_4$ titration, the results were so high as

to suggest a withdrawal of oxalate from solution; this effect being more apparent where there was an adsorptive surface, *i.e.* in the case of non-deproteinised serum.

Conclusions.

(1) Owing to errors associated with adsorbed organic matter and incomplete precipitation, calcium estimations (by $KMnO_4$ or acidimetric methods), effected on non-deproteinised serum and C.S.F., cannot be relied upon to give absolute values, or values such that significance can be attached to minor variations.

(2) The above errors may be eliminated by adopting the method of Halverson and Bergeim [1917] as modified by Peters and Van Slyke [1932]; including the Halverson and Bergeim washing with removal of the mother-liquors by suction, as modified by Van Slyke and Sendroy [1929].

(3) Fatty and opaque sera can be successfully analysed by the above method.

I wish to thank Dr F. L. Golla, Director of the Central Pathological Laboratory, and Dr S. A. Mann for their advice and criticism. I am also indebted to the medical officers of the London County mental hospitals for their kindness in providing the samples.

REFERENCES.

Clark and Collip (1925). *J. Biol. Chem.* **63**, 461.
Halverson and Bergeim (1917). *J. Biol. Chem.* **32**, 101.
Heiduschka and Schmidt-Hebbel (1932). *Biochem. Z.* **253**, 336.
Hendriks (1929). *Biochem. J.* **23**, 1207.
Hirth and Klotz (1923). *Compt. Rend. Acad. Sci.* **192**, 1053.
Kramer and Tisdall (1921). *J. Biol. Chem.* **47**, 475.
von Meysenbug, Pappenheimer, Zucker and Murray (1921). *J. Biol. Chem.* **47**, 529.
Peters and Van Slyke (1932). Quantitative clinical chemistry, **1**, 767.
Stanford and Wheatley (1925). *Biochem. J.* **19**, 710.
Trevan and Bainbridge (1926). *Biochem. J.* **20**, 423.
Van Slyke and Sendroy (1929). *J. Biol. Chem.* **84**, 217.

XLVIII. A NEW SYNTHESIS OF 2-THIOLHISTIDINE TOGETHER WITH EXPERIMENTS TOWARDS THE SYNTHESIS OF ERGOTHIONEINE.

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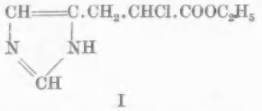
(*Received February 5th, 1933.*)

During the past year or two the problem of the synthesis of ergothioneine has received considerable attention in this laboratory. One mode of attack, which, whilst it failed in its ultimate object, led incidentally to the synthesis of 2-thiolhistidine has already been described [Ashley and Harington, 1930]. The work of these authors led immediately to an attempt to obtain ergothioneine by an analogous synthesis, namely by the benzoylation of ethyl α-chloro-β-glyoxaline-4 (or 5)-propionate (I) followed by conversion of the product (II) into ethyl α-chloro-γ-keto-δ-benzamidovalerate (III); it was hoped that the latter might be hydrolysed to the aminoketone (IV) and this converted into α-chloro-β-2-thiolglyoxaline-4 (or 5)-propionic acid (V) which, with trimethylamine, might be expected to yield ergothioneine (VI).



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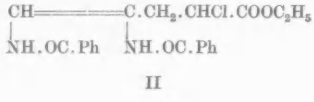

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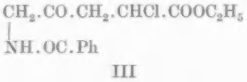

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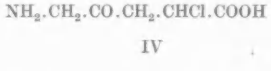

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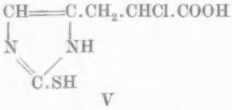

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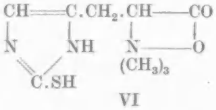

VI

All attempts to benzoylate ethyl α-chloro-β-glyoxaline-4 (or 5)-propionate have however proved fruitless. The conditions of the Bamberger fission of substituted glyoxalines are not very clearly established; according to Windaus, Dörries and Jensen [1921] the reaction does not occur with glyoxaline derivatives which contain a free carboxyl group in the side-chain, but succeeds with the esters of such derivatives; in no case however can the glyoxaline ring be ruptured by benzoylation if a carbonyl group be directly attached to it in the 4 (or 5) position, as for instance in glyoxaline-4 (or 5)-formaldehyde or ethyl glyoxaline-4 (or 5)-carboxylate. It appears that the presence of the negative halogen atom

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in the side-chain of ethyl α-chloro-β-glyoxaline-4 (or 5)-propionate brings this compound into the class of glyoxaline derivatives which are resistant to fission by benzoylation.

From time to time various other lines of attack have been tried and these are briefly outlined here in order to avoid unnecessary duplication by other workers.

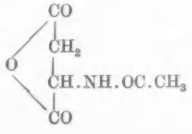
(*a*) One of the chief difficulties in working with 2-thiolglyoxalines consists in the lability of the sulphur atom which is removed with the greatest ease by oxidising agents. It was thought that this difficulty might be avoided by working with the corresponding disulphides and attempts were therefore made to oxidise 2-thiol-4 (or 5)-aminomethylglyoxaline [*cf.* Pyman, 1911] with iodine, the object being to substitute the amino-group of the disulphide which should result successively by hydroxyl and halogen and then to condense the product with ethyl sodiochloromalonate. The action of iodine on 2-thiol-4 (or 5)-aminomethylglyoxaline did not proceed smoothly however; a complex mixture of products was obtained which could not be readily purified, and this line of experiment was not pursued further.

(*b*) Phenoxyacetyl chloride gives, with diazomethane, a good yield of phenoxychloroacetone; the latter can be converted into phenoxyiodoacetone and this in turn, by condensation with hexamethylenetetramine and subsequent hydrolysis according to the method of Delépine [1895], yields phenoxyaminoacetone hydrochloride. Treatment of the latter with one molecular equivalent of sodium thiocyanate gives 2-thiol-4 (or 5)-phenoxyglyoxaline. This compound cannot however be satisfactorily hydrolysed to 2-thiol-4 (or 5)-hydroxymethylglyoxaline nor can the phenoxy-group be directly substituted by bromine with concentrated hydrobromic acid.

(*c*) A theoretically possible method of building up the type of compound necessary for the synthesis of an α-halogeno-β-glyoxaline-4 (or 5)-propionic acid by Gabriel's reaction consists in the condensation of an acylaminohalogenoacetone with ethyl sodiochloromalonate followed by liberation of the amino-group, formation of the thiolglyoxaline ring and hydrolysis and decarboxylation of the product. Very numerous attempts were made to condense phthalimidohalogenoacetones with ethyl sodiochloromalonate and indeed with ethyl sodiomalonate itself under various conditions, but all were in vain. The halogenoacetones reacted exothermically with the sodiomalonates with rapid elimination of the sodium halide, but normal condensation did not occur, the products consisting for the most part of highly pigmented resins. As a matter of fact the work to be described in the present paper shows that even if such a condensation had been successfully achieved the synthesis on these lines would have failed at a later stage.

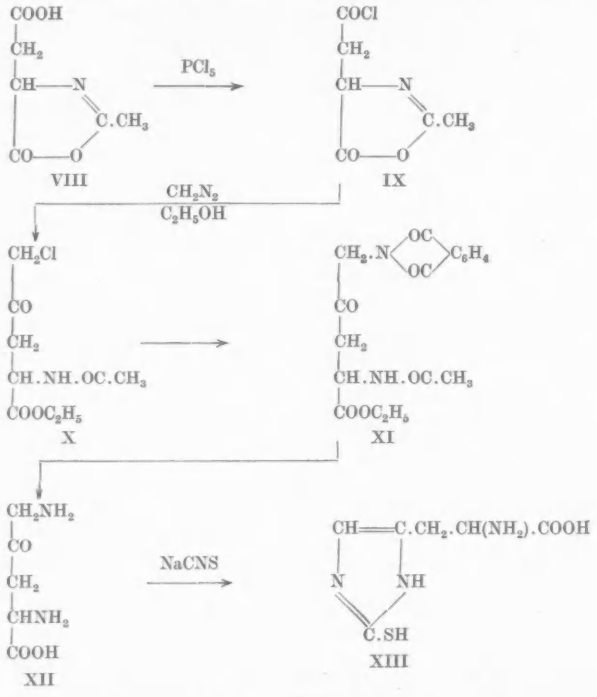
The object of the present paper is to describe an entirely different mode of attack. Although this has again failed to yield ergothioneine it has led to an independent synthesis of 2-thiolhistidine and is thought to be of sufficient chemical interest to be worthy of description.

Bergmann *et al.* [1926] described an anhydride resulting from the action of acetic anhydride on aspartic acid to which they ascribed the formula VII.



(VII)

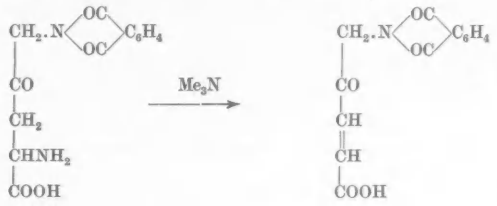
The experimental results upon which this formula was based were not conclusive however and could be equally well explained by making the more likely assumption that the compound was an azlactone (VIII); as will be seen from the experiments about to be described the main product of the action of acetic anhydride on aspartic acid or acetylaspartic acid is in fact this azlactone.



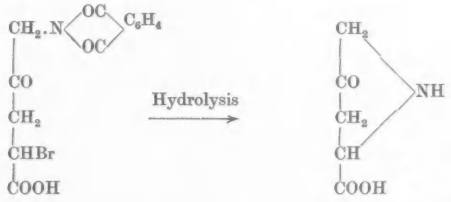
When the acetylation product (VIII) is treated with phosphorus pentachloride in acetyl chloride an acid chloride (IX) is produced; the action of diazomethane in dry ethereal solution on IX gives a product which, on dissolution in alcohol, yields ethyl α-acetamido-γ-keto-δ-chlorovalerate (X). The latter can be readily converted into the phthlamido-derivative (XI) which is hydrolysed by boiling with 20 % hydrochloric acid to give the hydrochloride of αδ-diamino-γ-ketovaleric acid (XII). The action of one molecular equivalent of sodium thiocyanate on XII yields the hydrochloride of 2-thiolhistidine from which the free amino-acid (XIII) is readily obtained.

In the attempt to synthesise ergothioneine the above series of reactions was modified as follows. Owing to the relative firmness with which the phthalimido-residue is held it is possible to effect a partial hydrolysis of compound XI with liberation of the α-amino- and carboxyl-groups to give α-amino-γ-keto-δ-phthalimidovaleric acid (XIV), and we hoped, by methylation of this compound, followed by hydrolysis of the phthalimido-group and synthesis of the thiol-glyoxaline ring, to obtain ergothioneine.

In this hope we were disappointed however since all attempts to methylate XIV led only to liberation of trimethylamine and production of an unsaturated acid. It is evident that, as in the case of aspartic acid, the presence of a carbonyl group in the β-position relative to the carbon atom to which the nitrogen is attached renders the betaine highly unstable.



XIV



XV

As a last resort the amino-group of compound (XIV) was interchanged for bromine by the action of nitrosyl bromide, the object being to hydrolyse the resulting α-bromo-γ-keto-δ-phthalimidovaleric acid (XV) and use the product for the preparation of α-bromo-β-2-thiolglyoxaline-4 (or 5)-propionic acid, which should finally be treated with trimethylamine to give ergothioneine. In this instance we were defeated by the fact that hydrolysis of the bromo-acid (XV) even under the mildest conditions consistent with removal of the phthalimido-residue was accompanied by elimination of hydrogen bromide and ring-closure.

It appears to us therefore that the only reasonable hope of synthesising ergothioneine rests in the action of trimethylamine on an α-halogeno-β-2-thiol-glyoxaline-4 (or 5)-propionic acid and that such an acid will have itself to be obtained by synthesis from an appropriate thiolglyoxaline; that is to say the formation of the thiolglyoxaline ring cannot be postponed until the last stage. In view of the difficulties referred to above of working with thiolglyoxalines the prospects for a synthesis of this kind are not encouraging.

The 2-thiolhistidine obtained in the course of the present work differs from that prepared by Ashley and Harington [1930] in being optically inactive since racemisation of the aspartic acid occurs during the treatment with acetic anhydride; it closely resembles the *l*-acid however in its physical and chemical properties.

Experimental.

Acetylaspartic acid. (*a*) Aspartic acid (10 g.) was dissolved in 2*N* sodium hydroxide (82·5 cc.) and the solution was treated with acetic anhydride (15·3 cc.) and 2*N* sodium hydroxide (165 cc.); the acetic anhydride and sodium hydroxide were added alternately in small portions, the mixture being vigorously shaken and cooled under the tap after each addition. At the end of the reaction 6*N*

sulphuric acid (82·5 cc.) was added and the solution evaporated under diminished pressure. The residue was extracted several times with boiling alcohol and the combined alcoholic extracts, after addition of a little water to inhibit esterification, were evaporated *in vacuo*. The syrupy residue set to a crystalline mass after keeping in an evacuated desiccator for several days; yield 9·5 g.

(*b*) Aspartic acid was dissolved in 2*N* sodium hydroxide (2 equivalents) and ketene was distilled into the solution until the latter was acid to litmus; 5*N* sulphuric acid (2 equivalents) was then added and the solution was evaporated under diminished pressure. The further working up was as described above.

The acetylaspartic acid obtained by either of these methods was difficult to recrystallise satisfactorily, being exceedingly soluble in water, alcohol and acetic acid and very sparingly so in other organic solvents. It was obtained in micro-crystalline condition on cooling a very concentrated solution in hot water and melted at 142–143°. (Found: C, 40·7; H, 5·3; N, 7·8 %. $C_6H_9O_5N$ requires: C, 41·2; H, 5·1; N, 8·0 %.) The compound had $[\alpha]_{5461}^{20°} + 5·9°$ ($c = 2·552$ in water).

Azlactone (VIII). (*a*) Aspartic acid (10 g.) was suspended in freshly distilled acetic anhydride (50 cc.) and the mixture was boiled for 35 minutes. The solution was then cooled, filtered from a trace of undissolved material and concentrated as rapidly as possible in a good vacuum, the bath temperature not exceeding 60°. The partly crystalline residue was dissolved in hot ethyl acetate, and the solution, on cooling, deposited 5·5 g. (47 %) of crystalline material. After recrystallisation from ethyl acetate or acetic acid it formed hard prisms melting at 145–146°.

(*b*) Acetylaspartic acid (5 g.) was suspended in acetic anhydride (10 cc.) and the mixture was boiled; a clear solution was rapidly obtained and boiling was continued for 2 minutes after this had occurred. The subsequent working up was as described above and the yield and properties of the product were similar. (Found: C, 45·7; H, 4·6; N, 8·7 %. $C_6H_7O_4N$ requires: C, 45·8; H, 4·5; N, 8·9 %.)

If the acetylaspartic acid employed for the reaction were not perfectly dry and if the reaction were carried out at 95° in an oil-bath instead of at the boiling temperature, an *anhydride* was obtained in variable yield which was isomeric with the above-described azlactone; this second anhydride was much less soluble than the azlactone and crystallised from ethyl acetate in prisms melting at 175°. It seems probable that the higher-melting anhydride possesses the constitution VII. (Found: C, 46·1; H, 4·5; N, 8·9 %. $C_6H_7O_4N$ requires: C, 45·8; H, 4·5; N, 8·9 %.)

Acid chloride (IX). Perfectly dry and very finely powdered azlactone (5 g.) was suspended in acetyl chloride (30 cc.) and the mixture was treated with powdered phosphorus pentachloride (7 g., 5 % excess). On shaking for a few minutes practically everything passed into solution with slight evolution of heat, after which crystallisation of the product set in rapidly and was soon complete. The mixture was cooled in ice and salt and diluted with an equal volume of anhydrous ether, after which the precipitate was filtered off, with exclusion of atmospheric moisture, and washed with light petroleum. The yield of dry white excessively hygroscopic crystalline powder was 94 % of the theoretical. (Found: Cl, 20·1 %. $C_6H_6O_3NCl$ requires: Cl, 20·2 %.)

Ethyl α-acetamido-γ-keto-δ-chlorovalerate (X). The acid chloride freshly prepared as described above from 5 g. of azlactone was suspended in anhydrous ether (250 cc.). The suspension was cooled in ice and diazomethane from 9 cc. of nitrosomethylurethane was passed into it, the mixture being continually agitated. The addition of the diazomethane was accompanied by vigorous evolution of nitrogen and dissolution of most of the solid. 15 minutes after the last of the diazomethane had been added the pale yellow solution was decanted

from a small amount of undissolved material, chilled in ice and salt and saturated with hydrogen chloride. As soon as the yellow colour due to the excess of diazomethane was discharged a voluminous white precipitate (presumably α-acetamido-γ-keto-δ-chlorovaleryl chloride) began to separate. Addition of hydrogen chloride was interrupted when this precipitate no longer increased in amount. Alcohol (80 cc.) was then added which caused immediate dissolution of the precipitate; if the operation had been properly conducted the solution was colourless at this stage. Solvents were evaporated under diminished pressure, overheating being carefully avoided and the last traces of alcohol being removed in a vacuum desiccator. The partly crystalline residue so obtained was rubbed up with cold aqueous sodium carbonate to neutralise traces of hydrochloric acid and the almost colourless crystalline precipitate was collected.

The product could be crystallised from hot water, in which it was easily soluble, or from toluene or xylene; it formed colourless leaflets melting at 128°. The yield was 2·25 g. or 32 % calculated on the azlactone employed. (Found: C, 46·0; H, 6·0; N, 6·0; Cl, 15·0 %. $C_9H_{14}O_4NCl$ requires: C, 45·8; H, 6·0; N, 6·0; Cl, 15·1 %.)

Ethyl α-acetamido-γ-keto-δ-phthalimidovalerate (XI). The chloroketo ester (X) (4·5 g.) was dissolved in anhydrous xylene (9 cc.) and to the solution, heated in an oil-bath at 135°, there was added freshly prepared potassium phthalimide (3·45 g.); heating was continued for 45 minutes. On cooling, the contents of the tube set to a crystalline mass; this was pulverised, freed from xylene and thoroughly washed with cold water.

The product was recrystallised from boiling 80 % alcohol from which it separated, on cooling, in fine white needles melting at 175°. The compound was readily soluble in alcohol; in water it was fairly soluble at the boiling temperature but very sparingly so in the cold.

The yield was 5 g. or 76 % of the theoretical. (Found: C, 59·0; H, 5·1; N, 8·1 %. $C_{17}H_{18}O_6N_2$ requires: C, 59·0; H, 5·2; N, 8·1 %.)

αδ-Diamino-γ-ketovaleric acid monohydrochloride (XII). Ethyl α-acetamido-γ-keto-δ-phthalimidovalerate (5 g.) was boiled under reflux for 2·5 hours with 55 cc. of 20 % hydrochloric acid. The solution was cooled and the phthalic acid which had separated was filtered off, the last traces being removed by extraction with ether. The solution was then concentrated to a low volume under diminished pressure and transferred to a vacuum desiccator over potassium hydroxide; removal of the last traces of hydrochloric acid in this way left a crystalline residue of the hydrochloride.

The compound was recrystallised from 60 % alcohol from which it separated in colourless non-hygroscopic prisms. It was extremely soluble in water and sparingly so in alcohol. On heating it turned yellow at 175° and blackened without melting properly at 210°.

The yield was 3 g. or 95 % of the theoretical. (Found: C, 33·0; H, 6·1; N, 14·8; Cl, 19·7 %. $C_5H_{11}O_3N_2Cl$ requires: C, 32·9; H, 6·0; N, 15·3; Cl, 19·5 %.)

dl-*2-Thiolhistidine*. αδ-Diamino-γ-ketovaleric acid hydrochloride (2·8 g.) was dissolved in water (3 cc.) and heated in a boiling water-bath. Sodium thiocyanate (1·3 g.) was added in three portions at 20-minute intervals and heating was continued for 1 hour after the last addition. The solution was then treated with saturated aqueous sodium acetate until no longer acid to Congo red, whereupon the amino-acid began rapidly to crystallise; after keeping overnight in the ice-chest the precipitate was collected and amounted to 1·5 g. (62·5 % of the theoretical). When recrystallised from hot water (charcoal) it formed pale straw-coloured plates of irregular shape similar in appearance to the *l*-2-thiolhistidine

synthesised by Ashley and Harington [1930]. (Found: C, 38·5; H, 4·9; N, 21·8; S, 16·9 %. $C_6H_9O_2N_3S$ requires: C, 38·5; H, 4·8; N, 22·4; S, 17·1 %.)

The product gave a ninhydrin reaction; in aqueous solution it decolorised alkaline permanganate instantaneously in the cold. With diazobenzenesulphonic acid and sodium carbonate it gave an orange colour which deepened in intensity and acquired a purplish tinge on addition of concentrated aqueous sodium hydroxide.

On evaporation of a solution of the amino-acid in concentrated hydrochloric acid in a vacuum desiccator over potassium hydroxide there was obtained a *dihydrochloride* which formed large colourless prisms, melting at 204–206° (decomp.).

When the amino-acid was oxidised with ferric sulphate under conditions precisely similar to those described by Ashley and Harington [1930] for the oxidation of *l*-2-thiolhistidine it gave a satisfactory yield of *dl*-histidine. No doubt remains therefore as to the constitution of the synthetic amino-acid.

α-Amino-γ-keto-δ-phthalimidovaleric acid (XIV). Ethyl α-acetamido-γ-keto-δ-phthalimidovalerate (2·5 g.) was boiled under reflux for 3 hours with *N* hydrochloric acid (25 cc.). On cooling the solution a small amount of phthalic acid separated; this was filtered off and the mother-liquor was thoroughly extracted with ether; the total amount of phthalic acid recovered was 0·3 g. corresponding with 25 % of the starting material. The aqueous solution was then neutralised by the addition of 2*N* sodium carbonate (12·5 cc.) when the aminoketophthalimidovaleric acid separated.

The precipitate was collected and recrystallised from hot water from which it separated in colourless needles melting at 165–166° (decomp.). (Found: C, 56·3; H, 4·8; N, 9·7 %. $C_{13}H_{12}O_5N_2$ requires: C, 56·5; H, 4·35; N, 10·15 %.)

All attempts to methylate this compound whether in the ordinary way with methyl sulphate and potassium hydroxide or with methyl iodide and anhydrous sodium carbonate in acetone (a mild method which has been employed with success in the preparation of the betaine of phenylalanine) led only to liberation of trimethylamine and formation of an unsaturated acid.

α-Bromo-γ-keto-δ-phthalimidovaleric acid (XV). α-Amino-γ-keto-δ-phthalimidovaleric acid (1·38 g.) was dissolved in 3*N* sulphuric acid (8·3 cc.) and potassium bromide (2 g.) was added. The solution was cooled in ice and vigorously stirred with a mechanical stirrer whilst finely powdered sodium nitrite (0·525 g.) was added in small portions during 20 minutes. When about half the nitrite had been added the bromo-acid started to crystallise. At the end of the reaction the product was collected and recrystallised from water; it formed fine colourless needles, melting at 185–186° (decomp.). (Found: C, 46·3; H, 3·2, N, 4·3; Br, 22·9 %. $C_{13}H_{10}O_5NBr$ requires: C, 45·9; H, 2·9; N, 4·1; Br, 23·5 %.)

The hydrolysis of this compound proceeded with extreme ease, 1 hour's boiling with 2*N* hydrochloric acid sufficing to liberate all the phthalic acid; whether the hydrolysis were conducted under these mild conditions however, or more vigorously with the employment of concentrated acid, the solution, after removal of the phthalic acid and evaporation under diminished pressure, yielded an oily residue, not easily soluble in water and non-basic in character.

REFERENCES.

Ashley and Harington (1930). *J. Chem. Soc.* 2586.
Bergmann, Stern and Witte (1926). *Liebig's Ann.* **449**, 277.
Delépine (1895). *Compt. Rend. Acad. Sci.* **120**, 501.
Pyman (1911). *J. Chem. Soc.* **99**, 668.
Windaus, Dörries and Jensen (1921). *Ber. deutsch. chem. Ges.* **54**, 2745.

XLIX. A MICRO-PHOTOMETRIC METHOD FOR THE DETERMINATION OF FREE CHOLESTEROL AND CHOLESTEROL ESTERS IN BLOOD-PLASMA.

By EDGAR OBERMER and REGINALD MILTON.

(*Received February 10th, 1933.*)

Existing methods for the separate analysis of free cholesterol and cholesterol esters in the blood-plasma are not suitable for routine micro-estimation on large numbers of specimens.

Almost all methods used in chemical pathology laboratories utilise the Liebermann-Burchard colour reaction. When figures arrived at by such methods have been compared with results of the digitonin technique, they have been found much higher. Various attempts have been made to overcome this difficulty. When these attempts have appeared successful, this has been due to incomplete extraction.

It is now known that these high figures are due to the fact that the Liebermann-Burchard colour reaction is given by a variety of substances in plasma extracts, apart from cholesterol.

In our search for a micro-method, we were unable to use a gravimetric technique. Unless large quantities of plasma are used, the results are unreliable.

Using the micro-digitonide method of Okey [1930], with subsequent sulphochromic oxidation, we were not able to arrive at consistent results owing to:

(*a*) the uncertainty of the oxidation procedure;

(*b*) the difficulty of ensuring a cholesterol digitonide precipitate free from extraneous organic matter.

We were also unable to arrive at consistent results with an attempted nephelometric method—re-precipitation of the digitonide from pyridine solution with sodium hydroxide.

We were therefore obliged to utilise a colour reaction. The insolubility of the digitonide in chloroform prevents use being made of the Liebermann-Burchard reaction, to which there would be no objection after the cholesterol had been separated in a pure state. Any modification of this reaction using solvents other than chloroform, while possibly giving the colour reaction, does so under conditions which are not susceptible of control.

In a recent publication of Bernoulli [1932], it is observed that the reaction of Tschugaew [1909] is applicable to both free cholesterol and cholesterol esters. The reaction consists of the addition of acetyl chloride to a glacial acetic acid solution of cholesterol in the presence of zinc chloride. On heating, a rose colour results, which finally becomes golden brown.

Discussion of Bernoulli's colour reaction.

Bernoulli has found that the reaction is given by other acid chlorides, particularly benzoyl and nitrobenzoyl. Obviously this reaction cannot be used directly upon blood-filtrates, which no doubt contain many products which would give the reaction, owing to their unsaturated nature.

For instance, a suspension of starch in glacial acetic acid gives a colour reaction at a very much faster rate, highly suggestive of dehydration. The presence of similar bodies in blood-filtrates, or blood-extracts, would no doubt have the same effect.

Further, Bernoulli has shown that although cholesterol esters give the reaction, they do so at a different speed and with less intensity than does free cholesterol. This would very much complicate any attempt at a direct estimation upon blood-extracts. We have found that cholesterol digitonide gives a colour reaction, a mixture of the colours produced by cholesterol and by digitonin being obtained. With digitonin the reaction is possibly of a similar nature to that obtained with glucosides—possibly a colloidal solution of carbon is formed due to the intensive dehydrating conditions which are present. The rates at which cholesterol and digitonin give the colour reaction are similar, although the reaction with digitonin is continuous. Conditions can be found, however, under which the reaction is so slowed down as to allow of comparable findings under a given set of conditions. Acetyl chloride, as proposed by Bernoulli, has many disadvantages. The reaction involves treatment in a boiling water-bath, and acetyl chloride is volatile below this temperature. Thus, unless boiling under reflux in a ground glass apparatus is resorted to, it is difficult to regulate the amount of acetyl chloride present. Further, it is difficult to maintain the purity of the acetyl chloride, any alteration in the concentration of which naturally affects results.

Discussion of similar modified colour reactions.

Apparently all acid chlorides give this reaction to a greater or less degree, suggesting that primarily the reaction is simply due to acetyl chloride, which is formed in each case by the action of the acid chloride on glacial acetic acid. Benzoyl chloride gives a very intense reaction. The difficulties regarding purity, referred to above, are equally applicable in this case. Further, the benzoic acid, which forms during the course of the reaction, is relatively insoluble in glacial acetic acid, and precipitation complicates the colour matching.

We find the best reaction is given by *p*-nitrobenzoyl chloride. The acid from this, however, is even more insoluble than benzoic acid. By cutting down the amount of acid chloride used, precipitation of the corresponding acid is minimised. This however is undesirable in view of the fact that the speed of the reaction is proportionately diminished. But the reaction rate may be increased by increasing the amount of zinc chloride used. This involves utilising a large quantity of a very hygroscopic substance, the undesirability of which was observed during the investigation. Thus we were led to adopt the use of *o*-nitrobenzoyl chloride. The colour reaction with this is less intense than in the case of the *p*-compound, but the corresponding acid produced during the reaction is completely soluble under the conditions chosen.

For a given solution of cholesterol digitonide, the intensity of colour developed is proportional to the amount of acid chloride present, the amount of zinc chloride present and the duration of boiling.

The effectiveness of zinc chloride is considerably impaired in the presence of even small traces of moisture. Complete freedom from moisture is ensured by dissolving the zinc chloride (3·5 %) in acetic acid.

With regard to the acid chloride, *o*-nitrobenzoyl chloride is solid at ordinary temperatures, but in view of its low melting-point it is easily manipulated with a pipette. The fact that it is normally in a solid condition gives stability, which is not found in the case of acetyl or benzoyl chloride. For practical purposes it may

be kept in glacial acetic acid solution for a period up to 1 week, without any obvious deterioration.

In utilising the colour reaction, we dissolved the digitonide precipitate in glacial acetic acid, containing 0·1 g. anhydrous zinc chloride, and used 0·2 g. of the acid chloride, diluting with glacial acetic acid to 5 cc. The time required for production of the colour has been found to be 50 minutes in a boiling water-bath, using the above concentration. Actually there is an increase in colour production over and above this time, but the rate is so slow that 10 more minutes in the bath produces only about 1 % increase in colour.

It is essential that all traces of moisture be excluded from the reaction. When the tube is placed in the boiling water-bath, the top should be covered, so that condensed water does not drop back into the tube. We have used a piece of glass tubing about 2 in. long, and wide enough to fit over the outside of the centrifuge-tube, drawn to a capillary at one end. This is placed over the tube and held in position by means of a piece of rubber tubing. All tubes and pipettes used during the colour-production stage of the estimation should be quite dry and all traces of moisture should be rigidly excluded.

Should a colorimetric end-point be resorted to, this is not so important, since the unknown and the standard would be subjected to the same variations in conditions.

Method of separation of digitonide.

Cholesterol is precipitated as digitonide from an alcohol-ether extract of blood, and the precipitate is removed by centrifuging. Transference from flask to centrifuge-tube is not quantitative if any of the usual wash solvents be used as a vehicle. We have used the technique of Yashuda [1931], which consists in boiling the dried contents of the precipitation flask with water, to dissolve excess digitonin, and then adding acetone to dissolve the emulsion of fat thus formed.

In this way quantitative transference of cholesterol digitonide is made. In view of the much diminished surface tension of fluids containing digitonin in solution, cholesterol digitonide is held in suspension to some degree, even after lengthy centrifuging. We have adopted the device of adding to the digitonide suspension a heavy flocculant substance, which will pack well and at the same time bring down with it all the cholesterol digitonide. After the first centrifuging this artifice need not be employed, since the excess of digitonin is removed and with it its reducing action on surface tension. Further washings with acetone and ether allow of a pure precipitate of cholesterol digitonide.

Method of hydrolysis.

In estimating total cholesterol, esters must be hydrolysed before precipitation as digitonide can occur.

Okey [1930] showed that the prolonged action of strong NaOH on cholesterol alters the molecule in such a way as to prevent the digitonide compound being formed. This was said to be due to an oxidative change and was prevented by passing nitrogen through the flask during hydrolysis. We have confirmed this destructive effect of hot alkali, but have been able to avoid the complicated technique of introducing nitrogen. If, after hydrolysis is complete, the alcohol is evaporated down to 2 or 3 cc., instead of to dryness, there is no fear of any oxidative change occurring. At this stage we dissolve out free cholesterol from the alkaline alcoholic solution with light petroleum.

We have found it unnecessary to acidify the hydrolysate before extraction is made; the smallest trace of acid inhibits the formation of the digitonide, whilst a small amount of alkali is without deleterious effect, in fact in its presence the precipitate comes down in a much more amorphous form, which aids effective centrifuging.

FINAL TECHNIQUE ADOPTED.

Solutions required:

(1) 3–1 alcohol-ether.
(2) Sodium ethoxide, 20 % in alcohol.
(3) Light petroleum.
(4) 1 % digitonin in 50 % alcohol.
(5) Acetone.
(6) 4 % aluminium chloride solution.
(7) Concentrated ammonia.
(8) 30 % hydrochloric acid.
(9) Ether.
(10) 3·5 % zinc chloride in glacial acetic acid. About 10 g. stick zinc chloride are weighed into a hard glass flask. The flask is heated over a free flame until all moisture and fumes are driven off and a clear melt is obtained. The flask is then rotated so that the melt distributes itself evenly over the sides of the flask. It is then allowed to cool down to below 100°. Glacial acetic acid is then added, and the flask is gently heated until solution is obtained. More glacial acetic acid is then added until the solution is 3·5 % with respect to zinc chloride.

This solution must be kept in a stoppered bottle and ordinary precautions taken to avoid absorption of moisture. The solution, even in this low concentration, is hygroscopic, and allowing it to stand in an open basin for a period of 1 hour has been sufficient to cause 50 % loss in its colour-producing power with regard to the reaction.

(11) 10 % *o*-nitrobenzoyl chloride in glacial acetic acid. This solution should be prepared by melting the solid acid chloride and diluting the liquid with glacial acetic acid. After 3 or 4 days the acid chloride tends to change over to the acid. Deterioration is not permissible to any large extent, since a reduction in the concentration of the acid chloride causes a slowing down of the colour reaction.

Technique.

Preparation of alcohol-ether extract. About 12 cc. of a mixture of 3–1 alcohol-ether are poured into a 20 cc. graduated flask. By means of a very fine pipette 1 cc. of plasma is delivered into the flask in a fine stream. The contents of the flask are well shaken, and it is then placed in a boiling water-bath until the contents begin to boil. The flask is then vigorously shaken and boiling continued for 3 minutes. At the end of this time the flask is removed from the bath and allowed to cool to room temperature. The contents are then made up to 20 cc. with alcohol-ether, vigorously shaken and filtered through a dried paper into a flask.

Free cholesterol. 10 cc. of alcohol-ether extract are evaporated to dryness with 0·5 cc. of 1 % digitonin solution. The last traces of alcohol are removed on the water-bath. 2 cc. water are added to the flask, the contents raised to boiling, and then the colloidal solution which forms is broken up by addition of 4 cc. acetone. The contents of the flask are transferred to a centrifuge-tube, 1 drop of aluminium chloride added, and after mixing, 1 drop of ammonia is used to precipitate aluminium hydroxide. The tube is then centrifuged at high speed for

about 1 minute. The supernatant fluid is completely decanted off. The precipitate is dissolved in 1 drop of 30 % HCl. The original precipitation flask is washed out with a further 3 cc. acetone, using a glass rod to aid removal of residual digitonide from the sides. The wash fluid is then added to the centrifuge-tube, the contents mixed and the whole again centrifuged for about 5 minutes. The supernatant fluid is decanted off, and the precipitate washed again with 2 cc. acetone. Finally the precipitate is washed with 3 cc. of ether, centrifuged, decanted, and the residual ether allowed to evaporate spontaneously.

·3 cc. zinc chloride-glacial acetic acid solution are added to the precipitate and then 2 cc. of acid chloride. The tube is covered and allowed 50 minutes in the boiling water-bath. At the end of this time the tube is allowed to cool and read in the Vernes photometer using the green screen.

Total cholesterol. 5 cc. of alcohol-ether extract are boiled under reflux for 30 minutes with 0·2 cc. sodium ethoxide.

At the end of this time the bulk of the alcohol is boiled off, and about 15 cc. of light petroleum are added. The contents are brought to the boil and vigorously shaken. 2 cc. of water are then added and the contents again thoroughly shaken. After settling, the light petroleum layer is poured off through a filter-paper into a dry flask. The residue is washed twice with 10 cc. light petroleum.

To the combined light petroleum extract is added 0·5 cc. digitonin solution, and the bulk of the light petroleum is distilled off.

10 cc. alcohol-ether are then added and the contents of the flask evaporated to dryness, finally removing the last traces of moisture on the water-bath.

After this the procedure is exactly as for free cholesterol.

Comparative figures.

	Macro-gravimetric technique of Gardner and Gainsborough [1927] (utilising 20 cc. plasma)	Suggested micro-photometric technique (utilising 1 cc. plasma)	Macro-gravimetric technique of Gardner and Gainsborough [1927] (utilising 20 cc. plasma)	Suggested micro-photometric technique (utilising 1 cc. plasma)
1. Total	161	166	242	245
2. Esters	110	112	169	169
3. Free	51	54	73	76
1. Total	232	228	246 247	241 247
2. Esters	154	145	163 161	159 158
3. Free	78	83	83 86	82 89
1. Total	206	198	194	196
2. Esters	136	130	126	129
3. Free	70	68	68	67
1. Total	189	194	225	225
2. Esters	129	134	139	146
3. Free	60	60	86	79
1. Total	254	248	214	220
2. Esters	175	178	130	139
3. Free	79	70	84	81

Calculation. In each case the optical density of the solution, as given by the Vernes photometer, is read off on a graph relating optical density to concentration.

The graph is prepared as follows. Standard solutions of cholesterol in chloroform, containing from 0·1 to 1·0 mg., are subjected to exactly the same technique as is used for free cholesterol.

The resulting densities of colour produced are plotted against concentration of cholesterol.

If the conditions employed when estimating unknown solutions of cholesterol are identical with those employed during the construction of the graph, the latter may be used for converting optical densities into concentration.

This technique has been worked out for adaptation to the Vernes-Bricq-Yvon Photometer [Obermer and Milton, 1932]. The same technique can of course be used for reading with any accurate photometer, such as the Zeiss "Stufenphotometer," and the Exton scopometer.

REFERENCES.

Bernoulli (1932). *Helv. Chim. Acta*, **15**, 274.
Gardner and Gainsborough (1927). *Biochem. J.* **21**, 130.
Obermer and Milton (1932). *J. Lab. Clin. Med.* **17**, 792.
Okey (1930). *J. Biol. Chem.* **88**, 367.
Tschugaew (1909). *Ber. deutsch. chem. Ges.* **42**, 4634.
Yashuda (1931). *J. Biol. Chem.* **92**, 303.

L. THE EFFECT OF DIET ON THE PLASMA-PHOSPHATASE OF SHEEP.

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(*Received February 10th, 1933.*)

Recent work on the rôle of phosphatase has made it clear that the enzyme is closely concerned in the metabolism of calcium and phosphorus and in bone formation. Thus, it has been shown that an increased plasma-phosphatase content accompanies certain bone diseases in human beings [Kay, 1930], that a rise in value also occurs during the healing of fractures [McKeown and Ostergren, 1931], and that, during the healing of rickets in children, the phosphatase values are still abnormally high even after the serum-Ca and blood-inorganic P have returned to normal [Smith and Maizels, 1932]. Bodansky and Jaffe [1931] observed that certain dietary deficiencies were accompanied by changes in the serum-phosphatase of rats.

In view of the marked changes which have been found in the serum-Ca levels of sheep suffering from experimentally produced "bent-leg" [Auchinachie and Fraser, 1932], it was considered desirable to determine whether the plasma-phosphatase of sheep fed on the same or similar diets would be affected in any way.

Experimental.

Blood samples were drawn at monthly intervals, and the plasma-phosphatase, serum-Ca, and blood-inorganic P values were determined. Plasma-phosphatase was estimated by the method of Kay [1930], serum-Ca by Tisdall's modification of the Kramer-Tisdall technique, and inorganic P by Havard and Reay's modification of Briggs's adaptation of the Bell-Doisy method. The sheep were also weighed at monthly intervals.

Exp. 1. It was obviously essential in an experiment of this nature and duration to consider the possibility of seasonal changes. During the course of the experiment the blood of normal sheep fed on adequate diets was analysed. The average results are given in Table I. It was not possible to get samples from

Table I. *Blood analyses of normal sheep at different months.*

					mg./100 cc.	
Month	Source	No of animals	Units phosphatase Av.	Range	Serum-Ca	Blood-inorganic P
March	Institute	4	0·264	(0·226–0·350)	10·58	6·71
June	Slaughter-house	11	0·335	(0·081–0·388)	9·48	4·22
October	Institute	34	0·255	(0·169–0·347)	10·42	5·79
November	Institute	4	0·283	(0·276–0·291)	10·20	5·11
December	Institute	37	0·226	(0·127–0·323)	11·12	5·22

normal sheep during the summer months, but blood from slaughter-house sheep was analysed instead. The rather high plasma-phosphatase values and low serum-Ca and blood-inorganic P, however, would appear to indicate that the slaughter-house animals were in a rather poor nutritional state.

It can be seen from these results that there is no evidence of a definite seasonal change in plasma-phosphatase. The differences noted in the subsequent experiments can, therefore, be attributed only to dietary factors and not to seasonal changes. Furthermore, as the sheep in these experiments were housed indoors, they were all the less likely to be affected by climatic differences.

Exp. 2. Four half-bred (Border Leicester × Cheviot) wether hoggs[1], strictly comparable as regards weight, age (7 months) and general health, were housed indoors and divided into two groups. The bedding consisted of moss litter, and dietary control was complete.

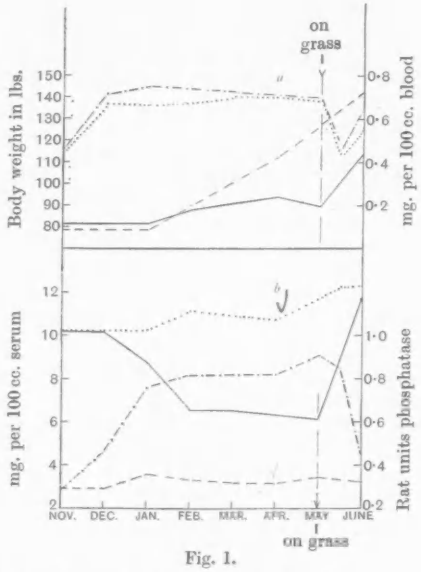

Fig. 1.

(*a*) Body weight in lbs. (complete ration) — — —
Body weight in lbs. (basal ration) ———
Inorganic P (complete ration) . . . . . .
Inorganic P (basal ration) —·—·—

(*b*) Serum-Ca (complete ration) . . . . . .
Serum-Ca (basal ration) ———
Phosphatase (complete ration) — — —
Phosphatase (basal ration) —·—·—

Group I was given the basal ration described by Auchinachie and Fraser [1932]. It was deficient in calcium but high in phosphorus when compared with good cultivated pasture, the ratio $CaO : P_2O_5$ being 1 : 4·17, whereas good pasture usually has a ratio of the order 1 : 0·77 (on a dry matter basis).

[1] Immature castrated males.

Group II received the basal ration supplemented with

(*a*) ground limestone (the quantity given, 0·5 oz. per sheep per day, raised the lime intake from 2·7 to 9·8 g. per day):

(*b*) cattle cod-liver oil, 2 cc. per sheep per day.

As will be seen from Fig. 1, definite changes in the three blood constituents studied were established after 2 months, and after 6 months they were much more marked. As the animals in Group I were then in very poor condition, the experimental feeding was discontinued, and the sheep in both groups were turned out to pasture, two further blood analyses being carried out at intervals of 14 days.

The average results of Exp. 2 are shown in Fig. 1, from which it can be seen that, 1 month after the experiment had commenced, the plasma-phosphatase content of the sheep in Group I showed a marked increase in value, unaccompanied by any appreciable change either in Ca, P or body weight. Indeed, it was only after 2 months that the serum-Ca had fallen appreciably relative to the original values, and it was a little after this that clinical evidence of malnutrition first became evident. The average plasma-phosphatase value in this group had, by this time, reached a level of 0·761 unit, nearly 3 times the original (0·283 unit), and it continued to rise slowly until it reached a value of 0·903 unit in May, when the artificial diet was discontinued. Except for a slight retardation, the Ca curve of Group II on the adequate diet is almost a mirror image of the phosphatase curve. The same negative correlation is seen to have occurred after the sheep were turned out to pasture, but the serum-Ca returned to normal levels, while the plasma-phosphatase still remained at a level higher than its initial value. In Group II neither of these constituents of the blood underwent any appreciable change except for a very slight fall in plasma-phosphatase and rise in serum-Ca after the sheep had been on pasture. The inorganic P showed no changes corresponding to changes in diet, for there was a rise in both groups during the experimental feeding. This increase was doubtless due to the high phosphorus content of the ration and therefore agrees with the result of the previous experiment (Auchinachie and Fraser). The weight curves bring out clearly the inadequacy of the basal ration (Group I) as compared with the supplemented ration, which appears to possess as good growth-promoting properties as good cultivated pasture.

Exp. 3. This experiment was begun for another purpose, and plasma-phosphatase determinations were begun only when it became evident from Exp. 2 that alterations in plasma-phosphatase content were likely to be brought about by deficient diets. Only two sets of values are, therefore, available, those for March and April.

Thirty-six healthy black-faced sheep, 7 months old, were divided into 6 equal groups which were placed on the diets outlined in Table II. Pre-experimental phosphatase values were not estimated, but those obtained as a standard of comparison in Exp. 2 can safely be employed as they refer to the same time of the year as that in which Exp. 3 was begun (*i.e.* November).

It will be seen that the Ca and phosphatase values obtained for the basal and basal + $CaCO_3$ groups agree very closely with those obtained for the basal group in Exp. 2, but the addition of $CaCO_3$ alone to the basal ration apparently checked to some extent the fall in serum-Ca and rise in plasma-phosphatase. The addition of 2·5 cc. of cod-liver oil (Group 3) to the basal ration was, however, of much greater benefit in this respect, the level of plasma-phosphatase being normal, although the serum-Ca values were slightly lower than those of sheep fed both $CaCO_3$ and cod-liver oil. It would appear that 2·5 cc. of cod-liver oil

Table II.

Group	Diet	March: Units of phosphatase	March: Range	mg./100 cc.: Serum-Ca	mg./100 cc.: Blood-inorganic P	April: Units of phosphatase	April: Range	mg./100 cc.: Serum-Ca	mg./100 cc.: Blood-inorganic P
1	Basal	0·699	(0·535–0·776)	6·29	7·50	0·727	(0·615–0·863)	6·36	7·59
2	Basal + $CaCO_3$ (2 %)	0·669	(0·543–0·814)	7·75	8·93	0·653	(0·564–0·777)	7·31	7·12
3	Basal + C.L.O. (2·5 cc. per sheep per day)	0·293	(0·182–0·385)	10·00	8·17	0·232	(0·189–0·315)	10·44	7·03
4	Basal + $CaCO_3$ + C.L.O. (1 cc. per sheep per day)	0·360	(0·274–0·436)	11·24	7·97	0·390	(0·192–0·536)	11·20	7·74
5	Basal + $CaCO_3$ + C.L.O. (2·5 cc. per sheep per day)	0·298	(0·216–0·389)	10·96	7·99	0·321	(0·272–0·388)	11·60	7·43
6	Basal + $CaCO_3$ + C.L.O. (5 cc. per sheep per day)	0·360	(0·243–0·424)	10·38	8·13	0·322	(0·262–0·461)	11·14	7·50

per sheep per day are more conducive towards a normal phosphatase value than either 1 cc. or 5 cc. Again, as in Exp. 2, the inorganic P values are higher than those of sheep fed on good cultivated pasture (unpublished data).

Exp. 4. Effect of pregnancy on plasma-phosphatase. In the course of our survey of the plasma-phosphatase content of the blood of various types of animals, it was found that the value in pregnant cows was low. This led to a more thorough study of the influence of pregnancy. In Table III are given the

Table III.

Class of animal	Number	Units of phosphatase
Sheep (pregnant)	56	0·066 (0·022–0·099)
Sheep (non-pregnant)	37	0·226 (0·127–0·323)
Cows (5 months pregnant)	6	0·130 (0·090–0·158)
Cows (8½ months pregnant)	6	0·069 (0·051–0·088)
Cows (non-pregnant)	11	0·146 (0·109–0·207)

values for cows and sheep during the later stages of pregnancy, together with those of non-pregnant animals. It would appear from these results that the plasma-phosphatase value is lower during pregnancy. This is not apparent in the data recorded by Kay [1930] for human beings but is in general a confirmation of the findings of Wilson and Hart [1932] for dairy cows.

Discussion.

It is clear from these experiments that disordered metabolism of Ca and P is associated with a marked rise in plasma-phosphatase. Thus, in Exp. 2, the value for sheep on the basal ration had risen from the pre-experimental level of 0·283 to 0·465 unit a month after the animals had been placed on a diet low in Ca and rich in P. This value went on increasing, at first rapidly and then more slowly, until a level of 0·903 unit was reached, when the deficient ration was withdrawn. It is important to note that the increase in plasma-phosphatase, following the inadequate diet, occurred appreciably earlier than the fall in serum-Ca, and, what is still more important, before any other sign of disordered metabolism had appeared. It would seem, therefore, that plasma-phosphatase estimations are of greater diagnostic value than the serum-Ca level or clinical observation in regard to the state of the Ca and P metabolism. This conception is supported by the fact that the plasma-phosphatase of the sheep on the deficient

diet fell less rapidly than the serum-Ca rose towards their respective normal levels when the sheep were put on pasture. This finding agrees with that of Smith and Maizels [1932] cited above.

Knowledge of plasma-phosphatase is not yet sufficiently far advanced to enable one to indicate all the factors concerned in maintaining it at normal levels. It is, however, obvious that something in cod-liver oil plays a large part in this regard; for, as Exp. 3 shows, the plasma-phosphatase level remained practically normal in those animals which received 2·5 cc. daily. It would also appear that an optimum amount of the oil is necessary, because the phosphatase level was nearer normal for animals receiving 2·5 cc. of cod-liver oil than for those which received either 1 cc. or 5 cc. daily. While it is probable that vitamin D is the chief beneficial factor in cod-liver oil, there seems some discrepancy between the effect on the metabolism of Ca and P caused by vitamin D supplied by cod-liver oil and by irradiated ergosterol preparations (unpublished data). Experiments to clear up this point are now in progress. The calcium content of the diet has also some effect on the mineral metabolism of the animal as judged by plasma-phosphatase, but to a much smaller extent than cod-liver oil.

The data available do not enable one to assess the significance of the low plasma-phosphatase in pregnancy in ruminants. It is probable that simultaneous estimations of foetal and maternal blood would throw some light on the matter.

Summary.

1. The plasma-phosphatase of the blood of sheep fed on a ration low in Ca and high in P increased progressively to from 3 to 4 times its normal value, while the serum-Ca fell.

2. Plasma-phosphatase determinations give a much earlier indication of disordered Ca and P metabolism than either serum-Ca, blood-inorganic P, general state of health or body weight.

3. Plasma-phosphatase appears to be lower in pregnant than in non-pregnant ruminants.

We desire to express our thanks to Prof. J. J. R. Macleod and Dr H. E. Magee for advice, and to Mr W. Thomson for tending the animals.

REFERENCES.

Auchinachie and Fraser (1932). *J. Agric. Sci.* **22**, 560.
Bodansky and Jaffe (1931). *Proc. Soc. Exp. Biol. Med.* **29**, 199.
Kay (1930). *J. Biol. Chem.* **89**, 235, 249.
McKeown and Ostergren (1931). *Proc. Soc. Exp. Biol. Med.* **29**, 54.
Smith and Maizels (1932). *Proc. Roy. Soc. Med.* **25**, 795; *Arch. Dis. Child.* **7**, 149.
Wilson and Hart (1932). *J. Dairy Sci.* **15**, 116.

LI. A NEW CONDUCTIVITY METHOD FOR THE DETERMINATION OF THE CELL VOLUME OF BLOOD.

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(*Received February 10th, 1933.*)

Many investigators [Bugarszky and Tangl, 1898; Stewart, 1899; Fraenckel, 1904; Gram, 1924; Fricke, 1924, 1, 2] have worked on the determination of the cell volume of blood by the conductivity method, improving that method but not succeeding in making it so accurate and simple that it could be commonly used. There is every reason to expect, however, that the conductivity method, so sound theoretically, when applied rightly will finally replace the haematocrit method, which is inaccurate and cannot be much improved.

The chief cause of the inexactitude of the conductivity method, in its present state, is the measurement of the conductivity of the whole blood. The blood as a suspension being often very unstable, its conductivity is also often very unstable; and yet it is necessary for accurate cell volume determinations to measure the conductivity of every sample of blood with the corpuscles each time in the same state of dispersion, if not in uniform dispersion, a condition which is very difficult to realise in practice. In a slowly settling blood, the corpuscles may be nearly equally dispersed while the conductivity is measured, but in a rapidly settling blood they are already agglutinated, vertically oriented and depositing; and these changes have a great influence on the conductivity. Stirring, besides being inconvenient, does not necessarily bring the corpuscles into a state of equal dispersion, neither does it always secure the same dispersion.

In the "two tubes" conductivity method, lately described [Slawinski, 1929; 1930], this inaccuracy is avoided by the measurement of settled blood, whose stability is much greater. A considerable simplification of the former method is also obtained by the use of two conductivity cells, instead of one, and the direct measurement of the ratio of two conductivities. This makes both the use of the thermostat and the necessity to separate the plasma for the measurement of its conductivity, as in the old method, unnecessary.

The conductivity cells are of the tube form, the only form practically indicated for the measurement of settled blood. A small conductivity tube, of about 0·5 cc. capacity, for human blood, is shown in Fig. 1.

The operations are conducted as follows.

Two conductivity tubes, closed at one of the ends with caps, are completely filled with blood from the same sample (by means of a dropping tube worked by a screw, for prompt and equal blood distribution between the two tubes), then closed at the other ends and placed, one tube horizontally and the other vertically,

in a holder, connected to a Wheatstone bridge. After a variable time (a few hours to 24), when the blood corpuscles have settled completely, we measure:

k, the ratio of the conductivity of the horizontal tube to the conductivity of the vertical tube, with the bridge;

h, the height of the blood deposit in the vertical tube, in relation to the height of the tube, with a telescope.

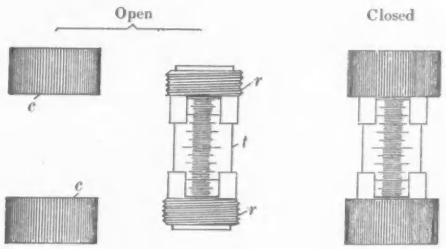


Fig. 1. Conductivity tube. t, graduated glass-tube; r, metallic rings, with threads, secured on the tube; c, metallic caps, with inner threads and platinum or platinized electrode bottoms, for the closing of the tube.

From these data the cell volume of blood is calculated by formula (4) given below.

The following formulae have been theoretically deduced for the method, and practically verified:

$$(1)\quad k_0 = \frac{\text{conductivity of plasma}}{\text{conductivity of dispersed blood}} = N + 1,$$

$$(2)\quad k_v = \frac{\text{conductivity of plasma}}{\text{conductivity of settled blood, in vertical tube}} = hN + 1,$$

$$(3)\quad k_h = \frac{\text{conductivity of plasma}}{\text{conductivity of settled blood, in horizontal tube}} = \frac{N+1}{(1-h)N+1},$$

$$(4)\quad k = \frac{\text{conductivity of settled blood, in horizontal tube}}{\text{conductivity of settled blood, in vertical tube}} = \frac{N^2}{N+1}h(1-h) + 1,$$

where

$$N = \frac{1{\cdot}6438 + 0{\cdot}1036\, w_s}{1 - w_s} w_s,$$

$$w_s = \frac{w}{h},$$

w, cell volume of entire blood, per unit volume of the blood;

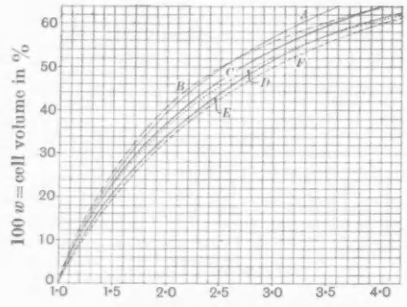
w_s, cell volume of the deposit of the blood corpuscles, per unit volume of the deposit;

h, height of the deposit in the vertical tube, in relation to the height of the tube.

The above function (1), as defined by the author, and similar functions k_0 of other investigators, are represented for comparison in Fig. 2.

The functions (2), (3) and (4), calculated for a cell volume $w = 0{\cdot}4$, are represented in Fig. 3.

The curves for settled blood show that the conductivities of both tubes, just filled with blood and placed in the holder, are the same, but as the blood cells are sinking the resistance of the vertical tube k_v augments, that of the horizontal tube k_h diminishes, and the ratio $k = \frac{k_v}{k_h}$ increases. After 24 hours k equals



$$k_0 = \frac{\text{conductivity of plasma}}{\text{conductivity of dispersed blood}}$$

Fig. 2. Dispersed blood conductivity curves.

Curve *A*, from Maxwell's [1892] theoretical formula for non-conductive suspensions.
" *B*, from Bugarszky and Tangl's empirical formula for the blood.
" *C*, from the author's theoretical formula for non-conductive suspensions.
" *D*, Gram's empirical curve for the blood.
" *E*, from Fricke's theoretical formula for the blood.
" *F*, Fraenckel's empirical curve for the blood.

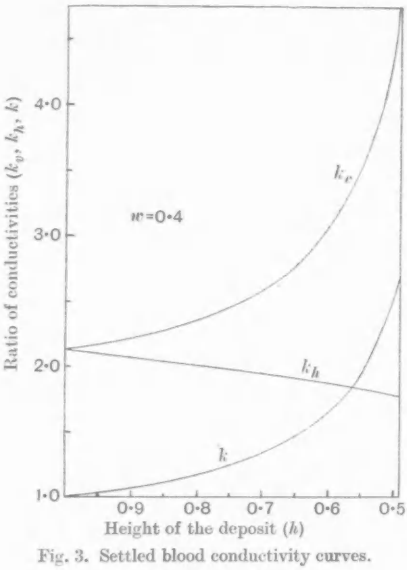


Fig. 3. Settled blood conductivity curves.

$$k_v = \frac{\text{conductivity of plasma}}{\text{conductivity of blood in vertical tube}},$$

$$k_h = \frac{\text{conductivity of plasma}}{\text{conductivity of blood in horizontal tube}},$$

$$k = \frac{\text{conductivity of blood in horizontal tube}}{\text{conductivity of blood in vertical tube}}.$$

from 2·5 to 3·0, in the case of human blood; in the case of horse blood, it equals 6 or more.

For simplification's sake, formulae (1) and (3) can be discarded: formula (1) since it is but a particular case of formula (2), when $h = 1$, and formula (3) as having, for the present, no practical application. The remaining formulae (2) and (4), which represent methods (2) and (4), may be expressed as follows:

$$(2) \qquad \frac{k_v - 1}{h} = N = \frac{1{\cdot}6438 + 0{\cdot}1036 \frac{w}{h}}{1 - \frac{w}{h}} \frac{w}{h},$$

$$(4) \qquad \frac{k - 1}{h(1 - h)} = n = \frac{N^2}{N + 1}.$$

Method (2) necessitates the use of a vertical tube filled with blood and a horizontal or vertical tube filled with plasma. The conductivities can be measured:

(*a*) When the cells are dispersed ($h = 1$), as in the old conductivity method. Since no thermostat is used, care should be taken that the temperature of both tubes, when the conductivity is measured, is the same. This method may be advantageously used in the case of a slowly settling blood.

(*b*) When the cells are well settled. This method will be seldom employed, having no practical advantages over method (4), given below, which is simpler.

Method (4) is the most advantageous in the case of promptly and moderately promptly settling blood. It necessitates, as said already, the use of two tubes filled with blood, one horizontal and one vertical, and the measurement of the conductivities when the cells are well settled. This method is very accurate with horse blood, and with human blood when the precipitation is accelerated. It is less exact with slowly settling blood, the concentration of the deposits (w_s) being probably in that case somewhat uneven, greater at the bottom of the deposits than at the top. In the case of very slowly settling blood, it is more advantageous to measure the conductivity of dispersed blood, using method (2).

For the calculation of the cell volume (w) by formula (2), we calculate N, as a function of k_v and h and then find w as a function of N and h; we calculate similarly when formula (4) is used. Special tables make these calculations easy.

The conductivity tubes must be of accurate make, to allow an exact determination of the height of the blood deposit, the accuracy of this measurement being important. The line of separation of the red corpuscles is usually very sharp, when viewed by artificial light falling on the deposit from behind and from above. It is this line, and not the line of the white cells, often indistinct, that should be read. The determination of the ratio of the conductivities does not need to be so accurate when method (4) is used; but it should be most exact when dispersed blood is measured by method (2). One must remember that the temperature of both tubes must be the same at every measurement of their conductivity; hence this measurement should not be made just after standing for some time near the holder, or after any other heating of the tubes. The first condition of accuracy is, however, to have blood well protected against coagulation, to mix it well and to distribute it promptly between the two tubes. The latter is most important, of course, with rapidly settling blood.

The "two tubes" method can be applied to the accurate determination of the stability of cell suspensions [Slawinski and Pakowski, 1930; Labendzinski and Slawinski, 1931] and to the study of the permeability of the cell walls, by the measurement of the cell conductivity [Slawinski, 1930; Labendzinski and Slawinski, 1931]. As it allows a 0·1 % accuracy of the cell volume deter-

minations, it can be advantageously used to study the variations of the cell volume of blood in plasmata of different tonicities and for the calculation of the free water volume in the blood cells. Until now, most of these determinations have been made by the haematocrit method, which is inaccurate, and very different values have been obtained. Using the "two tubes" method, this determination becomes easy, and it has been found lately that the free water volume in horse blood cells is nearly 50 % of the cell volume[1].

A further application of the same method would be to study the concentration of ions in the cells and in the plasma, the distribution of urea between cells and plasma and the like.

SUMMARY.

This method measures the conductivity of settled blood, which permits a more accurate determination of cell volume than is possible by the measurement of unsettled blood by the old conductivity method. The simultaneous use of two conductivity cells enables the conductivity measurements to be made at air temperature and renders the use of the thermostat and the separation of the plasma for the measurement of its conductivity unnecessary. By these simplifications the conductivity method becomes almost as simple as the haematocrit method and has the advantage of being much more accurate than the latter.

The cells are of tubular shape and closed at their ends by metallic caps, which play the rôle of electrodes. They are entirely filled with portions of the same blood and placed, one horizontally and the other vertically, in a holder, connected to a bridge. The measurement is made after the blood corpuscles have settled completely (a few to 24 hours). The ratio of the conductivities of both tubes and the height of the deposit in the vertical tube are then measured. The cell volume is calculated as a function of these data.

By this method the cell volume of blood can be determined with an accuracy of 0·1 %, which renders possible an exact calculation of the free water volume in the erythrocytes and an accurate estimation of the distribution of substances and ions between the cells and the plasma.

[1] A report on these determinations will appear in the Bulletin International de l'Académie Polonaise des sciences et des lettres. Classe de Médecine.

REFERENCES.

Bugarszky and Tangl (1898). *Arch. ges. Physiol.* **72**, 531.
Fraenckel (1904). *Z. klin. Med.* **52**, 470.
Fricke (1924, 1). *J. Gen. Physiol.* **6**, 375.
—— (1924, 2). *J. Gen. Physiol.* **6**, 741.
Gram (1924). *J. Biol. Chem.* **59**, 33.
Labendzinski and Slawinski (1931). *Compt. Rend. Soc. Biol.* **108**, 590.
Maxwell (1892). Electricity and magnetism, I, 440. (Oxford, 3rd edition.)
Slawinski (1929). *J. Chim. Phys.* **26**, 368.
—— (1930). *J. Chim. Phys.* **27**, 604.
—— and Pakowski (1930). *Compt. Rend. Soc. Biol.* **103**, 807.
Stewart (1899). *J. Physiol.* **24**, 356.

LII. ACID CHANGES IN MILK PRODUCED BY CERTAIN BACTERIA.

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(*Received February 17th, 1933.*)

Introduction.

The scientific literature concerned with the decomposition of milk by micro-organisms is very extensive, and reference to it demonstrates that the changes occurring have in most cases been correlated with variations in the acid content, although protein breakdown has, to a lesser extent, been studied. As a somewhat crude index of change, titration with alkali is very simple in application, and it is probably for this reason that it has been so widely adopted, and that, together with determinations of the hydrogen ion concentration, it has received more attention than any other available method.

In some cases a determination of a somewhat indefinite figure called volatile acidity has been used, but very rarely have even these two determinations—titratable acidity and volatile acidity—been carried out on one and the same series of samples. An unfortunate fact, too, is that this titratable acidity is often quoted as "lactic acid"; the serious error which this may entail has already been demonstrated [Lampitt and Bogod, 1930]. It is unnecessary to point out that it is impossible to define the course of any changes by determinations of the total "free" acids, and it would appear that only by determining a series of acids can a truer picture be obtained of the activity of specific organisms.

The present work serves to demonstrate the complexity of the acid changes which take place when organisms, chosen as representative of distinct groups, are inoculated into sterile milk, and is preliminary to a study of the mutual effect of the associative or symbiotic growth of certain bacteria, which has already received a certain amount of attention. The general methods adopted in this work have already been described by us [Lampitt and Bogod, 1932]. They consist of the determination of total acidity by normal and by electrometric titration, of p_H, of lactic acid, of citric acid and of free fatty acid. Their application to the study of progressive bacterial action in milk and the souring of milk under different temperature conditions has been reported, and it would appear that the use of such a system of investigation gives a clearer idea of the complexity of the action than is given by the less comprehensive scheme of analysis adopted, *e.g.* by Marshall [1920] and by Porcher and Lambert [1930] in their respective works on association of *B. subtilis* and *S. lactis*, or by Vas [1924] in his study of *L. bulgaricus* and *S. lactis*.

Comparatively modern work of a fundamental nature in this field is not too extensive, but among the workers whose investigations abut on that described herein may be mentioned Svanberg [1918; 1919] who studied the limiting values

for the p_H of milk under the influence of bacteria and also the velocity of growth of the bacteria under different p_H conditions. Thus, a p_H of 4·0 was found to be the limiting value and a range of p_H 5·5–6·4 the optimum for *S. lactis*. Baker and his co-workers [1920] showed the relation between bacterial growth and total acid production in milk. Sherman and Holm [1922], while studying the effect of sodium chloride on bacterial growth, found that *E. coli* failed to grow at p_H 4·8. Scheer [1922] used the p_H value in studying acid production by *E. coli* in buffered sugar solution and obtained a p_H of 5 in 20 hours, thereafter the acidity increased only slightly for 1 or 2 days, when acid production ceased. A study of the decomposition products of spore-bearing bacteria in heated milk by Lisk [1924] included the changes in p_H, titratable and volatile acidity; except in one case, an increase in titratable acidity was accompanied by a decrease in p_H. Cianci [1929] followed the progress of acid formation in 1 % lactose under the action of various bacteria including *S. lactis*, *E. coli* and *A. aerogenes* and also studied the action of *E. coli* in 1 % glucose broth. He found that p_H and acidity were constant after 7½ hours. As will be shown later, with milk as the medium, these values only become constant after a much longer period.

The formation of acids volatile in steam from milk has been subjected to investigation, notably by Hammer and his associates. With Bailey [1919] the volatile acid production of starters and organisms isolated from these was studied; with Cordes [1921] the relation of volatile acidity to titratable acidity as produced by *S. lactis* was found to be constant and the relationship to vary for different starters. Hucker [1928] suggested the use of the volatile acidity-titratable acidity relationship as a means of differentiating streptococci.

Citric acid utilisation by bacteria has received the attention of various workers. Kickinger [1922] reported that lactic acid-forming bacteria did not destroy citric acid, while peptonising bacteria did do so, and Koser [1924] found that typical coli of faecal origin were unable to affect sodium or potassium citrate, whereas the aerogenes-cloacae group all readily decomposed these salts. However, Hastings, Mansfield and Helz [1925] showed that citric acid fermentation depended upon the medium and found that the citrates in milk were not attacked by caseinogen-digesters, *S. lactis* or *A. aerogenes*, while *E. coli* had a marked positive effect.

Experimental.

The analytical methods used in this work have, but for one exception, been previously described [Lampitt and Bogod, 1932]. The exception, the volatile acidity, has been determined by a method described by Hammer and Sherwood [1923].

The bacteria (Bergey's nomenclature) used in the investigation were:

(1) *Streptococcus lactis*. This was obtained from the National Collection of type cultures and catalogued as *Streptococcus acidi lactici*, Thaysen, No. 662.

(2) *Alcaligenes faecalis*. From the N.C.T.C., catalogued as *Bacillus faecalis alcaligenes*, No. 415.

(3) *Aerobacter aerogenes*. From the N.C.T.C., catalogued as *Bacillus lactis aerogenes*, No. 418.

(4) *Escherichia coli*. From the N.C.T.C., catalogued as *Bacillus coli communis*, Houston 2, No. 692.

(5) *Bacillus subtilis*. From the N.C.T.C., catalogued as *Bacillus subtilis*, Hay, No. 85.

(6) A lipase-producing micrococcus of unknown species isolated from an air culture.

Certified milk was sterilised, and 700 cc. portions were measured into 1 litre Erlenmeyer flasks and inoculated with a culture of the bacteria in milk. Sufficient inoculum was added to give as nearly as possible 100,000 organisms per cc. The flasks were placed in a room thermostatically controlled at 30°. One sample was analysed immediately after inoculation and the remainder at intervals over a period of 2 weeks. For convenience in tabulation the determinations are recorded under letters as follows:

T. Titratable acidity, given as cc. 0·25*N* NaOH required per 100 g. of milk.
E. Electrometric titration, given as cc. 0·5*N* NaOH per 100 g. of milk.
H. p_H value.
L. Lactic acid as percentage content.
C. Citric acid as percentage of the original amount.[1]
F. Free fatty acids given as cc. 0·1*N* NaOH per 100 g. of milk.
V. Volatile acidity, given as cc. 0·25*N* NaOH per 100 g. of milk.

The detailed results obtained are given in the following tables.

Table I. The total acidity determinations as given by T., E. and H. follow the normal course and show the attainment of a constant figure after approximately 6 days' incubation. The lactic acid content also increases to a reasonably constant figure in the same time. The volatile acidity, showing a marked increase,

Table I. S. lactis.

Period of incubation (hrs.)	T.	E.	H.	L.	C.	F.	V.
0	7·7	3·6	6·40	0·018	100	0·40	0·7
15	12·8	6·4	5·75	0·125	37·5	0·60	2·6
24	18·7	8·5	5·50	0·240	21·5	0·70	3·8
28	21·7	10·6	5·05	0·320	6·3	1·10	3·9
40	22·6	10·7	5·20	0·400	3·9	0·70	3·9
64	23·8	11·7	5·00	0·460	None	0·90	4·3
75	24·7	12·2	4·95	0·560	,,	1·10	4·2
88	29·3	14·0	4·75	0·595	,,	0·90	4·8
99	32·3	15·6	4·70	0·580	,,	0·90	4·4
118	34·5	17·1	4·55	0·700	,,	0·90	4·6
144	33·5	15·3	4·60	0·610	,,	0·90	4·5
169	34·3	17·2	4·50	0·640	,,	1·10	4·4
192	32·5	17·0	4·55	0·560	,,	1·20	4·1
244	32·8	16·4	4·60	0·645	,,	1·20	4·5
285	34·2	16·4	4·65	0·635	,,	1·20	4·6
333	33·6	17·0	4·50	0·605	,,	1·10	5·0

does not, however, represent a large proportion of the total acidity. The citric acid was found to disappear after 2 days; this does not agree with the work of Hastings *et al.* [1925] who stated that the citrates in milk were not attacked by *S. lactis*; Kickinger [1922] also reported that lactic acid-formers did not destroy citric acid. Consequently this destruction of citric acid was confirmed by us on various occasions and further, in the communication previously referred to [1932] it was shown that *L. bulgaricus* which produced over 2 % lactic acid also destroyed citric acid in milk comparatively rapidly.

In order to determine any possible variation due to the initial bacterial concentration, lactic acid and citric acid were determined in two further series inoculated with *S. lactis* to give 10,000 and 1000 organisms per cc. respectively.

The progressive changes are shown in Table Ia.

[1] The citric acid content varied for each supply of milk, results obtained being: 0·168, 0·155, 0·128, 0·181, 0·173, 0·154, 0·149, 0·131 and 0·190 %. In order, therefore, to compare results as between one organism and another, the results are expressed as stated.

Table IA. *Effect of variation of initial bacterial concentration.*

Period of incubation hrs.	Lactic acid %		Citric acid %	
	10,000 *S. lactis* per cc.	1000 *S. lactis* per cc.	10,000 *S. lactis* per cc.	1000 *S. lactis* per cc.
0	0·012	0·012	100	100
10	0·011	0·012	98·4	98·9
20	0·139	0·035	37·0	56·9
24½	0·241	0·198	22·1	23·8
30	0·274	0·269	9·9	11·6
34½	0·357	0·321	2·8	4·4
39	0·389	0·379	None	None
50	0·446	0·412	,,	,,
70½	0·455	0·483	,,	,,
94½	0·552	0·531	,,	,,
119	0·560	0·570	,,	,,

Table IA. If curves are drawn side by side with those obtained with an initial concentration of 100,000 per cc. (Fig. 1), it is apparent that the effect of decreasing the initial count is merely to delay the commencement of chemical change since once the action has commenced, the alterations follow a definite typical course.

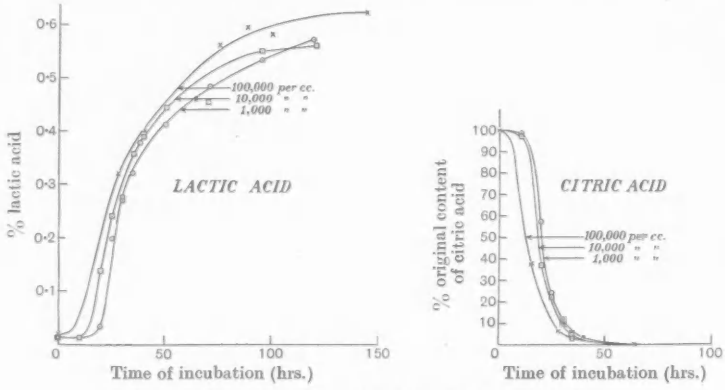

Fig. 1. Effect of variation of initial bacterial concentration.

Table II. B. faecalis alcaligenes.

Period of incubation hrs.	T.	E.	H.	L.	C.	F.	V
0	6·5	3·4	6·60	0·017	100	0·35	0·6
15	6·6	3·6	6·35	0·022	100	0·28	0·6
24	6·8	3·6	6·35	0·035	98	0·40	0·6
28	6·8	3·4	6·50	0·017	98	0·42	0·7
40	6·7	3·1	6·60	0·022	97	0·48	1·0
48	6·6	3·3	6·45	0·015	96	0·52	0·6
64	6·4	3·3	6·45	0·022	95	0·54	0·7
76	6·3	3·5	6·40	0·030	93	0·50	0·6
100	6·7	3·1	6·60	0·015	93	0·60	0·8
120	6·2	3·3	6·50	0·012	93	0·38	0·7
169	5·9	2·6	6·75	0·022	89	0·60	0·7
192	5·8	2·8	6·70	0·012	88	0·52	0·6
285	5·4	2·8	6·70	0·022	86	0·40	0·7
333	5·5	2·4	6·80	0·019	85	0·46	0·8

Table II. The values for T., E. and H. show the development of a slight alkalinity which follows a decrease in the citric acid content. Ayers *et al.* [1919] also demonstrated this increase in alkalinity with a decrease in the citric acid content. The values for L., F. and V. show no appreciable change.

Table III. The formation of acid, although not as great as with *S. lactis*, is considerable. Half of the citric acid content has been destroyed during the 2 weeks' incubation. It will be noted that volatile acid production proceeds comparatively rapidly to a maximum value and then steadily decreases.

Table III. Aerobacter aerogenes.

Period of incubation hrs.	T.	E.	H.	L.	C.	F.	V.
2	8·1	4·1	6·25	0·078	100	0·50	0·6
15	14·6	6·4	5·80	0·102	97	0·60	3·8
24	16·4	7·6	5·75	—	99	0·80	3·7
30	18·6	8·0	5·70	0·083	94	0·90	4·8
40	20·1	7·7	5·70	0·040	94	0·80	4·6
48	20·1	8·0	5·76	0·043	94	0·70	4·8
64	21·2	7·9	5·70	0·049	94	0·50	4·0
77	21·7	8·8	5·75	0·066	76	0·60	4·2
89	22·3	8·2	5·75	0·099	80	0·70	4·0
101	22·5	8·7	5·75	—	78	0·60	—
120	22·5	9·6	5·60	0·103	84	0·70	4·2
146	23·7	9·9	5·70	0·171	81	0·70	3·7
168	26·9	10·0	5·60	0·216	75	0·90	3·8
192	27·6	11·9	5·50	0·158	77	1·00	3·7
237	27·2	11·9	5·45	0·243	60	1·00	1·9
309	28·8	11·0	5·50	0·264	59	1·00	2·6
333	29·6	14·5	5·25	0·300	46	1·00	1·9

Table IV. Total acid formation here shows a rapid increase to a maximum value on the third day with little change thereafter. It will be noted that in this case the lactic acid content does not increase commensurately with the total acidity; indeed, the volatile acidity, of which the production is the highest of all those determined in this work, contributes more than the lactic acid to the total acidity. The destruction of citric acid is even more rapid than was the case with *S. lactis*.

Table IV. E. coli.

Period of incubation hrs.	T.	E.	H.	L.	C.	F.	V.
2	8·4	4·2	6·25	0·028	100	0·60	0·7
15	15·2	7·5	5·70	0·050	4	0·60	6·1
24	19·7	9·1	5·50	0·065	None	0·70	5·1
30	21·7	10·0	5·35	0·068	2	0·50	8·0
40	22·3	10·3	5·35	0·060	None	0·50	8·8
48	22·7	10·6	5·30	0·068	,,	0·50	8·3
64	26·0	10·9	5·30	0·065	,,	0·50	8·2
77	24·8	11·5	5·30	0·060	,,	0·80	8·3
88	25·7	11·3	5·30	0·055	,,	0·80	8·6
101	24·5	11·5	5·30	0·060	,,	0·80	8·6
120	24·1	11·8	5·30	0·070	,,	0·80	8·5
166	25·3	11·1	5·25	0·088	,,	1·00	9·2
192	23·7	11·8	5·30	0·060	,,	0·60	9·2
285	25·1	11·8	5·30	0·083	,,	0·70	8·5
333	25·7	12·5	5·20	0·073	,,	0·80	10·2

Table V. In considering the results in Table V, it should be remembered that the organism was existing virtually under anaerobic conditions. In the first place, during sterilisation, dissolved oxygen was driven out and later, when the inoculated milk was being incubated, a layer of cream formed on the surface.

It is obvious that there has been little action taking place in so far as the observed chemical characteristics are concerned. A count, taken at the end of the incubation period, showed however that the bacteria had increased from 100,000 to 1,200,000 per cc.

Table V. B. subtilis.

Period of incubation hrs.	T.	E.	H.	L.	C.	F.	V.
0	6·9	3·7	6·35	0·027	100	0·60	0·5
15	7·3	3·9	6·30	0·030	100	0·20	0·6
24	7·5	3·9	6·30	0·030	100	0·20	0·5
30	7·4	3·7	6·40	0·025	100	0·30	0·5
40	7·2	3·7	6·40	0·020	100	0·30	0·5
64	8·0	4·0	6·30	0·025	100	0·40	0·6
75	8·2	4·2	6·30	0·025	100	—	0·6
88	7·7	4·0	6·30	0·035	93	0·40	0·6
101	8·4	4·2	6·30	0·027	96	0·40	0·6
120	7·4	3·9	6·40	0·020	97	0·30	0·6
144	8·0	4·2	6·30	0·025	96	0·30	0·6
170	7·6	3·9	6·40	0·037	96	0·20	0·6
192	7·8	4·0	6·30	0·022	95	0·40	0·6
216	7·8	3·9	6·35	0·025	—	0·40	0·6
280	7·8	4·0	6·35	0·018	89	0·50	0·6
338	8·0	4·0	6·40	0·032	87	0·50	0·6

A further series inoculated with *B. subtilis* was investigated and precautions were taken thoroughly to shake the flask twice daily. In this manner it was ensured that the cream layer was broken up and dispersed through the milk, also that some air was dissolved. The results obtained are given in Table V A.

Table V A. It will be observed that in these circumstances the total acidity and lactic acid have been increased to some extent, but the effect on citric acid is comparable with the standard series: p_H and volatile acidity are again unaffected.

Table V A. B. subtilis (*shaking at intervals during incubation*).

Period of incubation hrs.	T.	H.	L.	C.	V.
0	6·6	6·30	0·018	100	0·6
98	7·2	6·30	0·028	92	0·8
147	8·2	6·50	0·060	89	0·9
194	9·6	6·45	0·086	88	0·9
288	10·8	6·40	0·081	84	0·9

Table VI. *Lipase-producing* Micrococcus *of unknown species.*

Period of incubation hrs.	T.	E.	H.	L.	C.	F.	V.
0	7·0	3·5	6·35	0·018	100	0·90	0·3
15	7·0	3·7	6·30	0·030	100	1·00	0·3
24	6·6	3·4	6·40	0·028	100	1·10	0·2
29	6·8	3·7	6·25	0·030	100	1·10	0·2
40	8·1	3·8	6·40	0·028	100	1·20	1·0
50	6·6	3·4	6·50	0·020	100	1·00	0·6
64	6·8	3·8	6·25	0·020	100	1·00	0·6
76	7·1	3·8	6·30	0·030	100	1·00	0·9
88	6·9	4·1	6·15	0·020	100	0·90	0·8
119	7·6	4·0	6·15	0·040	100	1·10	1·0
144	8·4	4·3	6·10	0·033	100	1·10	1·3
170	8·3	4·1	6·15	0·040	100	1·20	1·1
192	7·6	4·2	6·15	—	97	1·30	1·4
216	7·4	4·3	6·15	0·028	96	1·30	1·4
278	8·2	4·4	6·10	0·045	96	1·30	1·6

Table VI. Here again a decided progressive action is not apparent; a more marked formation of fatty acids and volatile acids is, however, to be observed. The bacterial count at the end of the incubation period was 101,000,000 as compared with the original 100,000 per cc.

The graphs presented show the relationships existing among the various bacteria in regard to each specific determination. The electrometric titrations closely resemble the results for titratable acidity and have therefore not been graphically represented.

Fig. 2. The relative total acid-forming properties of the bacteria under test existing under the same temperature conditions are well contrasted. An apparent resemblance between *E. coli* and *A. aerogenes* is shown.

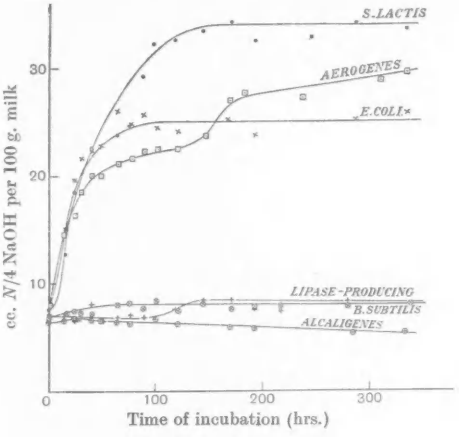

Fig. 2. Titratable acidity.

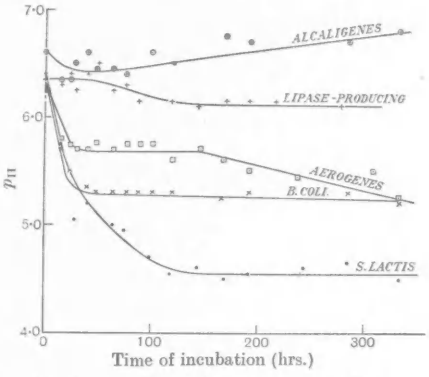

Fig. 3. p_H. *B. subtilis*—no apparent change.

Fig. 3. The picture presented by these curves resembles that given by the previous one in regard to the relationships between organisms.

Fig. 4. A decided difference between the action of *E. coli* and *A. aerogenes* is here demonstrated and the chief characteristic of *S. lactis* is amply shown.

Fig. 5. A further distinction between *E. coli* and *A. aerogenes* is the destruction of citric acid, exceedingly rapid in the case of *coli* and with *aerogenes* only

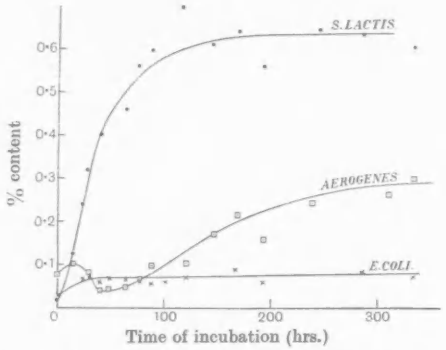

Fig. 4. Lactic acid. *B. subtilis*, lipase-producing and *alcaligenes*—no appreciable change.

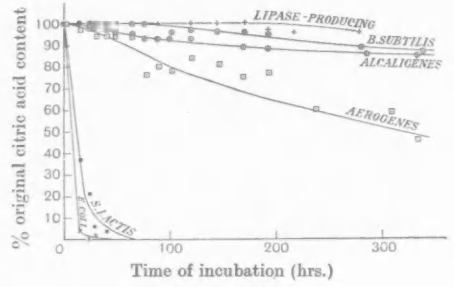

Fig. 5. Citric acid.

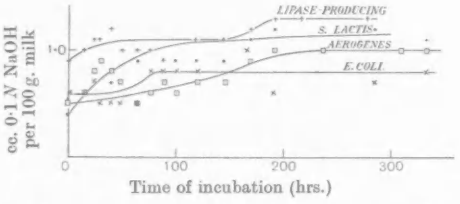

Fig. 6. Fatty acid. *B. subtilis* and *alcaligenes*—no appreciable change.

decreasing to about half the normal content. The citric acid loss with *B. subtilis* is very small, a fact which agrees with the work of Hastings *et al.* [1925] concerning the inactivity of caseinogen-digesters in connection with the citrates in milk, although Kickinger [1922] reports to the contrary.

Fig. 6. The free fatty acid formation, which refers to the higher fatty acids, is greatest in the case of the lipase-producing *Micrococcus*.

Fig. 7. *E. coli* is shown to produce more of the volatile acids than any of the other micro-organisms tested. It will be noted that there is an apparent relation-

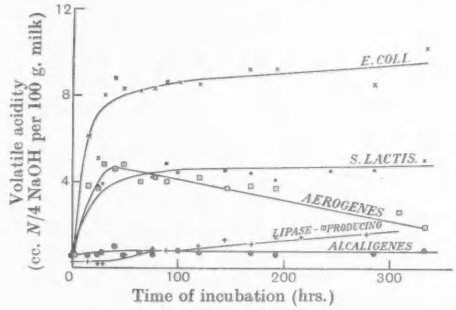

Fig. 7. Volatile acidity. *B. subtilis*—no appreciable change.

ship between the destruction of citric acid and volatile acid formation, since *coli* with the highest volatile acid production most rapidly destroyed the citrates; *S. lactis* is next and *A. aerogenes* is third in both respects.

We acknowledge with thanks the assistance of Messrs D. H. F. Clayson, D. G. Pirie and Miss D. A. Rudland in preparing cultures and performing inoculations and of Mr W. C. Wise for assistance with the analytical work.

Our thanks are also due to the Directors of Messrs J. Lyons and Co., Ltd., in whose laboratories this work was carried out, for permission to publish these results.

REFERENCES.

Ayers, Rupp and Johnson (1919). *U.S. Dep. Agric. Bull.* No. 782.
Baker, Brew and Conn (1920). *N.Y. Agric. Exp. Sta. Bull.* No. 74.
Cianci (1929). *Boll. Soc. Ital. Biol. Sper.* **4**, 1129.
Cordes and Hammer (1921). *Iowa Agric. Exp. Sta. Res. Bull.* No. 66.
Hammer and Bailey (1919). *Iowa Agric. Exp. Sta. Res. Bull.* No. 55.
—— and Sherwood (1923). *Iowa Agric. Exp. Sta. Res. Bull.* No. 80.
Hastings, Mansfield and Helz (1925). *Proc. Soc. Amer. Bact.* (Abstr. from *J. Bact.* **11**, 77.)
Hucker (1928). *N.Y. Agric. Exp. Sta. Tech. Bull.* No. 143.
Kickinger (1922). *Biochem. Z.* **132**, 210.
Koser (1924). *J. Bact.* **9**, 59.
Lampitt and Bogod (1930). *Chem. Ind.* Special No. March, 510.
—— —— (1932). *Chem. et Ind.* **27**, 3 *bis*, 777.
Lisk (1924). *J. Bact.* **9**, 1.
Marshall (1920). *J. Dairy Sci.* **3**, 406.
Porcher and Lambert (1930). *Le Lait*, **10**, 641.
Scheer (1922). *Biochem. Z.* **130**, 535.
Sherman and Holm (1922). *J. Bact.* **7**, 465.
Svanberg (1918). Inaug. Diss. 4. Stockholm, *Abst. Bact.* **4**, 284.
—— (1919). *Z. physiol. Chem.* **108**, 120.
Vas (1924). *Le Lait*, **4**, 625.

LIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXVII. THE PRODUCTION OF LUTEIC ACID FROM VARIOUS SOURCES OF CARBON BY *PENICILLIUM LUTEUM* ZUKAL.

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(*Received February 21st, 1933.*)

Raistrick and Rintoul [1931] isolated from the metabolic products of *Penicillium luteum* Zukal, when grown on an aqueous medium consisting only of glucose and inorganic salts, a mucilaginous, laevorotatory material which they termed luteic acid. This substance, the salts of which form very viscous solutions, is a colloidal material of high molecular weight, built up of polysaccharide units, which in the typical molecule arise from the condensation, with the loss of 2 molecules of water, of 2 molecules of glucose with 1 molecule of malonic acid in such a way as to leave only one carboxyl group of the malonic acid free. On mild alkaline hydrolysis luteic acid gives rise to a neutral laevorotatory polysaccharide which was named luteose, and which, on acid hydrolysis, gives glucose as the sole product of hydrolysis.

Luteic acid has up to the present been obtained solely from glucose as substrate, but in view of the unexpected results recorded by Coyne and Raistrick [1931] in obtaining mannitol from various sugars by the agency of the unnamed white species of *Aspergillus*, Catalogue No. Ac 55 (Thom and Church, No. 4640·489), it became of interest to determine whether the mould *P. luteum* Zukal would convert other sugars than glucose into the polyglucose complex, luteic acid, or whether each sugar employed as substrate would afford a different polysaccharide. It was hoped that the results obtained would throw some light on the initial stages of carbohydrate metabolism by moulds, and in particular by the species studied.

The organism used was a non-ascosporic form of *P. luteum* Zukal, Catalogue No. Ad 30, which was originally obtained from the Centraalbureau voor Schimmelcultures at Baarn in May 1925. It is the same strain as that described by Thom [1930, p. 448] as *P. luteum* Zukal, No. 11.

The medium employed in this work consisted of the usual Czapek-Dox salts, together with one of the following sources of carbon, in amount equivalent in carbon to 5 % glucose: fructose, galactose, mannose, xylose, arabinose, glycerol, citric and succinic acids. Acetic and pyruvic acids were also tried, but the mould did not grow on these substrates. The medium had the following composition: $NaNO_3$, 2 g.; KH_2PO_4, 1 g.; KCl, 0·5 g.; $MgSO_4,7H_2O$, 0·5 g.; $FeSO_4,7H_2O$, 0·01 g.; water to 1000 cc., with the addition of one of the sources of carbon mentioned, in amount equivalent in carbon to 50 g. of glucose. In the

case of the media containing the different carbohydrates or glycerol, the p_H was 4·2. In the case of citric acid and succinic acid sufficient 2*N* NaOH was added before final dilution with water to bring the p_H of the medium approximately to 4·2.

The medium was distributed in quantities of 350 cc. in 1 litre flasks, which were sterilised by steaming on each of 3 successive days and then inoculated with a spore suspension of *P. luteum* Zukal which had been grown on Czapek-glucose agar. The flasks were incubated for about 30 days at 24°. Very good characteristic growth was obtained with all the carbohydrates tried and also with glycerol. The growth obtained with succinic and citric acids was moderately good, being better with the former than with the latter. The metabolism solution was filtered from the mycelium, which was well washed with hot water, the washings being added to the main filtrate. The filtrate was then evaporated *in vacuo* to about 1/20 of the original volume and transferred as completely as possible to a beaker. After stirring in 1 cc. of concentrated HCl per flask of culture medium, four volumes of absolute alcohol were added, whereupon the polysaccharide was precipitated as a sticky mass.

The crude material at this stage contains a considerable amount of ash owing to its tendency to salt formation. Attempts were made to purify it by dissolving it in water and subjecting the solution to electro-dialysis. These attempts, however, were unsuccessful since the product appeared to be decomposed under the action of the electric current, breaking up into smaller units which passed through the membrane.

It was therefore decided to adhere closely to the original method of purification [Raistrick and Rintoul, 1931], which consisted in the precipitation of the aqueous solution acidified with HCl by means of absolute alcohol. After five such precipitations the product usually contained only a small amount of ash. The last precipitation was undertaken from neutral solution. The product was ground up in a mortar under alcohol to reduce it to a powdery consistency, filtered, washed with alcohol and finally with a little dry ether. After drying in a vacuum desiccator the product, which now consisted of a white friable powder, was subjected to analysis according to the methods described below.

The following determinations were made.

1. *Rotation of luteic acid.* (Table I, column 2.)

The specific rotation of the metabolism product was determined at 20° in 1 % solution using the mercury green line λ 5461. In order to obtain this value, it was necessary to neutralise the product with NaOH as described by Raistrick and Rintoul [1931]. In any case, owing to experimental difficulties, this figure can only be regarded as an approximation.

2. *Titration.* (Column 3.)

0·25–0·5 g. was titrated in the cold with *N*/10 NaOH, from which the equivalent was calculated.

3. *Ash.* (Column 4.)

The ash was determined on 0·1–0·15 g.

4. *Rotation of luteose.* (Column 5.)

The residual solution from the titration of luteic acid (item 2) was treated with 20 cc. of *N* NaOH and left overnight. 20 cc. of *N* HCl were added, and the neutral polysaccharide was precipitated with 4 volumes of absolute alcohol. The precipitate was allowed to settle, the supernatant liquid was poured off, and the precipitate was dissolved in water. After two further precipitations the luteose was separated and dried. Its specific rotation was determined in 1 % solution in a jacketed tube at 80° using the mercury green line λ 5461.

5. *Sugar determination.* (Columns 6 and 7.)

Acid hydrolysis. 1·0 g. of the metabolism product was treated with 50 cc. N H_2SO_4 and heated in a boiling water-bath under reflux for 9 hours. The volume was then made up to 100 cc. The sugar in the hydrolysate was determined by the polarimeter and by the Wood-Ost method. For the Wood-Ost determination 20 cc. of the hydrolysate were placed in a 25 cc. flask, and the volume was made up to the mark with $2N$ NaOH. In this way a practically neutral solution was obtained. 10 cc. were used for each Wood-Ost determination.

6. *Titration.* (Column 10.)

10 cc. of the hydrolysate from item 5 were treated with 5 cc. of N NaOH to neutralise exactly the mineral acid used in hydrolysis. The subsequent titration to phenolphthalein with $N/10$ NaOH then gave the organic acid produced during hydrolysis. This was expressed in terms of the weight of substance taken for hydrolysis as the "apparent equivalent" after hydrolysis. As it represents the difference between two large titrations it is subject to a large experimental error.

7. *Isolation of the acid produced on hydrolysis.* (Column 11.)

The remainder of the hydrolysate from item 5, about 70 cc. in volume, was extracted with ether for about 6 hours in a continuous extraction apparatus. The ether solution was poured into a sublimation tube, the ether evaporated and the residue sublimed in a high vacuum. The sublimed product was tested for malonic acid by determination of the M.P. and mixed M.P. with an authentic sample and by determination of the equivalent.

8. *Osazone from sugar.* (Columns 8 and 9.)

The extracted liquid was neutralised with $2N$ NaOH to phenolphthalein and filtered, and 2 cc. of glacial acetic acid and 3 cc. of phenylhydrazine were added. The mixture was heated in boiling water for about 30 minutes, allowed to cool, filtered, and the osazone separating washed with water, then with a little alcohol and finally with dry ether. When dry, 0·1 g. of the product was dissolved in 3 cc. of alcohol and 2 cc. of pyridine, and the optical rotation was determined immediately and after standing for 24 hours, using the mercury green line.

In some cases the solution did not transmit enough light for accurate determination of its optical rotation without recrystallisation. This was accomplished by cautious addition of water to the alcohol-pyridine solution.

The results obtained for the metabolic products isolated from the different sources of carbon are given in Table I. The figures given for luteic acid produced from glucose are extracted from Raistrick and Rintoul's paper [1931].

Considering now the results obtained for the hexoses, pentoses and glycerol, it is obvious that the figures obtained agree well with the values for luteic acid obtained from glucose as determined by Raistrick and Rintoul [1931], and it may be safely asserted that all these substances give rise to a malonylpolyglucose. Presumptive evidence for the presence of glucose as the only sugar is obtained from the close agreement of the glucose determinations by polarimeter and by the Wood-Ost method. The preparation of the phenylosazone and the determination of its characteristic initial and final rotations afford further evidence. In the case of the product from fructose, 5 g. were hydrolysed by heating with 250 cc. of N H_2SO_4 for 9 hours, and after extraction of the malonic acid the residual solution was neutralised with barium hydroxide. The barium sulphate was removed by filtration, and the filtrate was evaporated *in vacuo* almost to dryness. It was taken up in methyl alcohol, filtered and again evaporated. After redissolving in methyl alcohol, glucose crystallised on standing. It was recrystallised from methyl alcohol, weighed 1·85 g., now melted at

Table I. *Identification of luteic acid from various substrates.*

	Luteic acid			Luteose	Products of acid hydrolysis: Glucose				Apparent equivalent by titration	Equivalent of malonic acid isolated
					By polarimeter	By Wood-Ost	Osazone			
	Sp. rotation $[\alpha]_{5461}^{20°}$	Titration equivalent	Ash %	Sp. rotation $[\alpha]_{5461}^{20°}$	g./100 cc.	g./100 cc.	Initial rotation	Final rotation		
Glucose	−47°	435	0·79	−46·4° (at 90°)	0·840	0·846	−0·87° (calc.)	—	222	51·6
Fructose	−41°	768	0·87	−46°	0·841	0·843	−0·90°	−0·43°	407	51·7
Galactose	−38°	601	3·62	−46°	0·782	0·795	−0·86°	−0·42°	307	52·0
Mannose	−37°	817	3·46	−45°	0·756	0·769	−0·85°	−0·42°	322	51·8
Xylose	−37°	839	2·33	−43°	0·700	0·727	−0·72°	−0·44°	382	51·9
Arabinose	−37°	772	3·85	−35°	0·833	0·795	−0·80°	−0·42°	361	52·5
Glycerol	−30°	489	1·03	−46°	0·817	0·820	−0·79°	−0·42°	265	52·2
Succinic acid	−41°	8370	0·70	—	0·908	0·902	−0·72°	−0·39°	2630	—
Citric acid	−27°	2000	3·26	−32°	0·760	0·778	—	—	662	—
1	2	3	4	5	6	7	8	9	10	11

144–148° and gave no depression when mixed with authentic glucose. The osazone was prepared and the rotation determined as usual. The actual isolation of glucose from this particular product affords final proof of the formation of glucose from sugars other than glucose.

The production of malonic acid on hydrolysis of the metabolic product is equally definite, since in each case it was actually isolated by ether extraction, purified by sublimation, and its mixed M.P. and equivalent determined. There was in no case any depression of M.P. when the product was mixed with authentic malonic acid, and the equivalent as determined corresponds in each case very closely with the theoretical figure of 52.

The proof that the malonylpolyglucose obtained is identical with luteic acid rests upon the comparison of the figures obtained for the metabolic products from the various sugars with those given by the product from glucose. Though there are certain divergences here, they can be satisfactorily explained. The difficulties in obtaining the specific rotation on the original material, owing to its difficult solubility and the great viscosity of the neutralised solution, make this figure only an approximation. In Table I the values given, though somewhat variable, are of the same order and sign and agree sufficiently well in view of the above explanation.

The titration figures vary widely, and it seems quite certain that in the prolonged purification to which the product is subjected it is to some extent hydrolysed, the extent of hydrolysis depending on the duration of the purification process. We desire to state here that we do not regard luteic acid as a chemical entity with a constant chemical composition and a constant molecular weight. Rather are we of the opinion that luteic acid is analogous with acetylcellulose and nitrocellulose, in that although the ultimate products of hydrolysis are invariably solely malonic acid and glucose, these compounds are not necessarily produced in fixed and unvarying proportions. On the other hand, we regard luteose as a chemical entity in exactly the same sense as cellulose may be so regarded.

In spite of variations referred to above, it will be observed that the equivalent of the product is approximately twice the "apparent equivalent" of the hydrolysed material, findings which agree with the presence in the metabolic products

of malonic acid having one free and one bound carboxyl group, the latter becoming titratable after hydrolysis.

The values for the specific rotation of the luteose obtained on alkaline hydrolysis agree sufficiently well with the figures obtained from authentic luteose, and in view of these facts it may be definitely stated that luteic acid is produced by *P. luteum* Zukal from glucose, fructose, galactose, mannose, xylose, arabinose and glycerol.

Coming now to the consideration of the products from succinic and citric acids, it will be observed that the equivalent is, in each case, very high, indicating that if luteic acid, having a relatively small equivalent, is produced from these substrates, it must have undergone considerable hydrolysis. Though it is possible that this may have occurred during purification as already explained, it appears much more probable that the hydrolysis is here due to an increase in the p_H of the medium during the course of the experiment owing to the consumption of the organic acids used as substrates. The final p_H values of the media for succinic and citric acids were 8·8 and 6·8. Since only mild alkaline conditions are necessary to eliminate completely the malonic acid from luteic acid, the product obtained from succinic acid would be expected to contain practically no malonic acid, as is seen to be the case. Citric acid occupies an intermediate position since, in this case, a very small amount of acid was extracted by ether after acid hydrolysis which after sublimation melted slightly below the M.P. of pure malonic acid, but gave no depression on mixing. The amount was too small for an equivalent determination, but it seems reasonably certain that the metabolic product in this case did contain malonic acid.

The production of glucose in each case is indicated by the agreement between the Wood-Ost and polarimeter figures, and is proved in the case of the succinic acid product by the production of the osazone. On the other hand, no osazone could be obtained from the citric acid product, as the acid hydrolysate, which was rather dark in colour, only gave rise to a small amount of a tarry product on heating with phenylhydrazine. Further, the specific rotations of the metabolic product and the neutral carbohydrate obtained from it by alkaline hydrolysis are abnormal, and hence the production of luteic acid from citric acid must be regarded as unproven, although it is fairly certain that a malonylpolyglucose is produced.

The most interesting feature of these observations is the demonstration of the power of the organism employed to convert all these varied materials into a polyglucose. Whether we assume that glucose is first formed and then condensed into a polysaccharide, or whether we suppose that the materials offered are first built up into polysaccharides containing their own type of unit, the complex then being in some way directly converted into the polyglucose, it seems remarkable that the interconversion of sugars, apparently so difficult of accomplishment by purely chemical synthesis, is performed with such ease by the mould. That such behaviour may not be by any means rare, however, in the metabolism of micro-organisms is indicated by the observation of Coyne and Raistrick [1931], who obtained mannitol from a whole series of sugars, *viz.* glucose, mannose, galactose, xylose and arabinose, by the agency of a white species of *Aspergillus*. Further, Haworth, Raistrick and Stacey [1932] isolated from the metabolic products of *Pencillium varians* G. Smith [1933], when grown on a medium containing glucose as the sole source of carbon, a polysaccharide the molecule of which consists of a chain of about eight hexose units. The chain is built up from one unit of glucose at the one end of the chain, one unit of either altrose or idose at the other end, and about six units of galactose placed intermediately.

The elucidation of the mechanism by which these changes are brought about must await further work. At present the most attractive hypothesis would appear to be the degradation of the source of carbon to relatively simple molecules containing only a few carbon atoms, which are then built up to form carbohydrates of the peculiar pattern required by the organism, acting as a template, much in the manner that ingested proteins are broken down in the animal body to the constituent amino-acids, which are then used for synthesising the proteins specific for the organism. It is as yet too early to speculate on the actual degradation products which are the precursors of the specific polysaccharides, although obviously they must be of a relatively simple nature, since they are derived from such a variety of substrates.

SUMMARY.

Luteic acid is elaborated by *Penicillium luteum* Zukal when grown in a synthetic medium, containing as sole source of carbon any of the following substances: glucose, fructose, galactose, mannose, xylose, arabinose and glycerol. Indications of a similar product were obtained with succinic and citric acids as substrates. Since luteic acid gives malonic acid and glucose on acid hydrolysis, proof has been afforded of the conversion by this organism of the hexoses fructose, galactose and mannose, of the pentoses xylose and arabinose and of other carbon compounds into glucose.

This work has been rendered possible by a grant to one of us (J. H. B.) from the Research Council of Imperial Chemical Industries Ltd., to whom we tender our best thanks.

REFERENCES.

Coyne and Raistrick (1931). *Biochem. J.* **25**, 1513.
Haworth, Raistrick and Stacey (1932). *Chem. Ind.* **51**, 980.
Raistrick and Rintoul (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 255.
Smith (1933). *Trans. Brit. Mycol. Soc.* **18**, Part 1. (In press.)
Thom (1930). The Penicillia. (Baillière, Tindall and Cox, London.)

LIV. INVESTIGATIONS ON THE DETERMINATION OF VITAMIN D BY THE PREVENTIVE METHOD.

THE PREVENTIVE DOSE ESTABLISHED AS CURATIVE.

By POUL SCHULTZER.

From the State Vitamin Laboratory, Copenhagen.

(*Received January 21st, 1933.*)

In a previous paper [Schultzer, 1931] I gave the result of some studies on the preventive method for estimation of vitamin D. Young rats were kept on a rachitogenic diet for 28 days, and then the degree of rickets was examined. With the object of using a shorter experimental period, I studied the course of rickets throughout the experimental period by means of frequent radiographic examinations of the same animal, as I had found that the information obtained in this way is sufficiently reliable. With this technique I was further enabled to make use of animals employed for other purposes.

The question is of theoretical as well as practical interest. When a young rat on rachitogenic diet receives a preventive dose of vitamin D, its bones are found at the end of the experiment to be quite normal histologically and chemically. The development of the animal has not been normal, however, its daily increase in weight being considerably below the normal. Thus, the preventive dose of vitamin D does not counterbalance in every respect the deficiencies of the rachitogenic diet. A suitable change in the Ca : P quotient prevents the development of rickets, and at the same time the rate of growth becomes normal [Schultzer, 1927].

Technique.

The technique employed in these studies was the same as described before. It may be emphasised, however, that in these frequent X-ray examinations of the knee it proved advantageous not to anaesthetise the animals. Bourdillon *et al.* [1931] report that 2 % of the animals died under ethyl chloride anaesthesia. With repeated X-ray examinations such a mortality would have reduced considerably the number of animals in this material.

Each animal was radiographed 8 times—on the 1st, 4th, 8th, 12th, 16th, 20th, 24th and 28th days. If one of these days happened to be a Sunday, the radiography was done on Saturday or Monday. On the 28th day the animals were killed by coal gas, and autopsy was performed.

The degree of radiographic rickets was estimated without scale readings. Bourdillon and Bruce's [1932] radiographic scale for prophylactic dosing was not considered serviceable in these studies, as it applies only to young rats of a definite age. Instead, the estimation was limited to a simple measurement of the width of the uncalcified epiphyseal zone with the aid of a magnifying glass

(× 4). As a rule, the measurement was easy to make, but at times it was rather difficult on account of blurring of the top of the metaphysis. I think, however, that the approximate error from this source usually comes within 0·1 mm. The measurement was always made at the same spot—not in the midline of the bone but a little to the side of this, towards the fibula, where the width of the zone is usually the greatest when the leg of the animal is placed as in these experiments (see Figs. 2 and 3).

A. *Negative controls.*

Throughout the experimental period the negative controls received a daily addition of 0·2 g. peanut oil (Fig. 1). The greatest width of the epiphyseal gap was found to appear on the 12th day, after which it diminished noticeably. At the end of the experiment, the autopsy showed marked rickets. The radiographs also showed pronounced rickets.

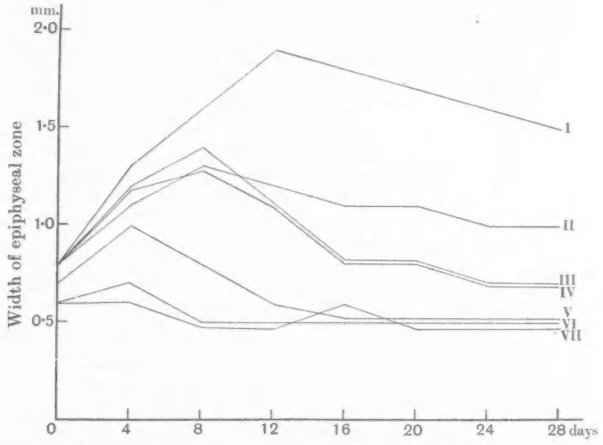

Fig. 1. Preventive dosing of cod-liver oil, including negative controls and normal young rats. I. Mean of 13 animals. Negative controls (0·2 g. peanut oil per day). Autopsy: 3 +. II. Mean of 6 animals. 1 mg. cod-liver oil per day. Autopsy: 1½ +. III. Mean of 9 animals. 2 mg. cod-liver oil per day. Autopsy: ½ +. IV. Mean of 12 animals. 3 mg. cod-liver oil per day. Autopsy: ½ +. V. Mean of 5 animals. 5 and 6 mg. cod-liver oil per day. Autopsy: −. VI. Mean of 5 animals. 10 and 20 mg. cod-liver oil per day. Autopsy: −. VII. Mean of 6 animals. Normal diet. Autopsy: −.

B. *Addition of cod-liver oil.*

The daily addition of cod-liver oil was given in doses of 1, 2, 3, 5, 6, 10 and 20 mg. in 0·2 g. peanut oil. It was the same cod-liver oil as was used in my experiments reported in 1931. It now turned out that 3 mg. was not a fully preventive dose as found before, about half of the animals showing signs of rickets on autopsy as well as on the final X-ray examination.

From the graphical presentation of the width of the epiphyseal zone throughout the experimental period (Fig. 1) it is evident that the maximum width of the zone is found on the 8th day with doses of 1, 2 and 3 mg., and on the 4th day with doses of 5, 6, 10 and 20 mg. On going into the data on the individual animals some surprising conditions are revealed. For example, 2 animals dosed

with 2 mg. did not show any sign of rickets at the end of the experiment, either on autopsy or on X-ray examination. In rat No. 3073 (Fig. 2) the epiphyseal zone was 0·6 mm. in width at the beginning of the experiment; 8 days later the width of the zone was 1·1 mm.; then it diminished, so that on the 28th day it was only 0·5 mm. In the case of rat No. 3433 (Fig. 3) the findings were even more striking. At the beginning of the experiment the epiphyseal zone was 1·0 mm. in width. It may be mentioned here that at this point of time—*i.e.* in normal rats 1 month old—the width of this zone has been found to vary from 0·5 to 1·0 mm. In one case (rat No. 3208) the width of this zone was even 1·2 mm., but in this case the measurement was very difficult to make on account of a

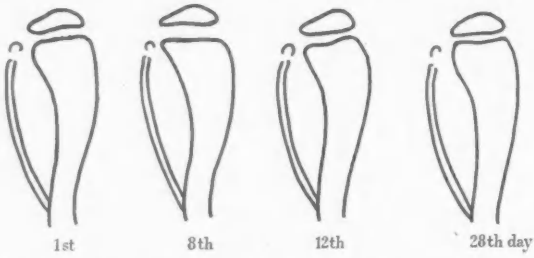

Fig. 2. Preventive dosing of cod-liver oil. Rat No. 3073. 2 mg. cod-liver oil per day. Autopsy – . Radiographic rickets on the 8th day. Pronounced healing on the 12th day. No demonstrable radiographic rickets on the 28th day.

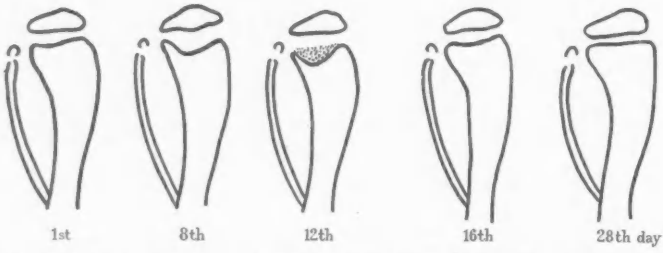

Fig. 3. Preventive dosing of cod-liver oil. Rat No. 3433. 2 mg. cod-liver oil per day. Autopsy: – . Radiographic rickets on the 8th day. Healing with calcium deposit—as with curative dosing—on the 12th day. No demonstrable radiographic rickets on the 28th day.

mishap in the photography. In rat No. 3433 the epiphyseal zone was 1·7 mm. wide on the 8th day, and 0·5 mm. at the end of the experiment. In this rat the diminution in the epiphyseal gap was introduced by deposition of lime salts in the lower part of the epiphyseal zone (see Fig. 3, 12th day), similar to the repair observed in the curative method. During the next 4 days this deposition of calcium had become part of the reconstruction of the top of the metaphysis, through which process the epiphyseal zone was further narrowed. The change taking place here corresponded fairly well with the difference between the scale Nos. 8 and 10 in the radiographic scale of healing of rachitic rats set up by Bourdillon *et al.* [1931].

From Fig. 1 it is evident that the curve changes in form with increase in the dosage of cod-liver oil. The curve for the 1 mg. dose is a little lower than the

curve for the negative controls. The initial rise is far less, the maximum being reached as early as the 8th day; then the curve falls slowly. With the 2 and 3 mg. doses the initial rises are about the same as with the 1 mg. dose, but having reached the maximum the curves fall more rapidly and are considerably lower than the preceding curve. The 5 and 6 mg. doses show even smaller initial rises; the 10 and 20 mg. doses show almost no initial rise.

Unfortunately the number of animals in this series is not large enough to give the same average values for the width of the epiphyseal zone in all the groups of doses at the beginning of the experiment; this detracts somewhat from the comparability of the results.

C. *Addition of standard preparation of vitamin D.*

Similar experiments were made cn rats with different doses of irradiated ergosterol, the standard preparation furnished by the National Institute for Medical Research. The doses are given in International units (Fig. 4). This series does not comprise as many rats as the preceding. On the whole, the findings in this series are similar to those above. The smallest dose, 0·05 I.U., gave a pronounced maximum on the 8th day, but the curve is plotted from the

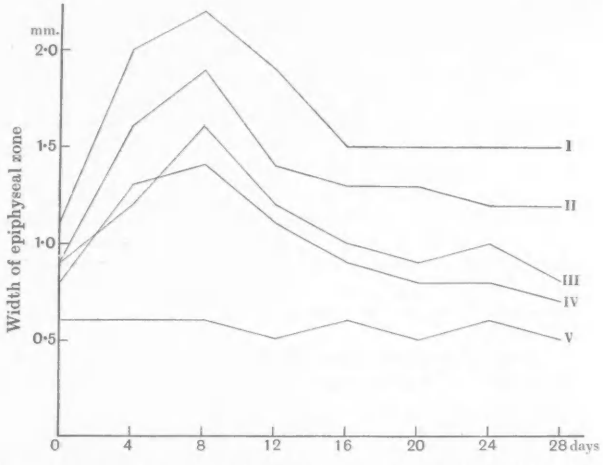

Fig. 4. Preventive dosing of vitamin D standard preparation. I. Mean of 2 animals. 0·05 I.U. per day. Autopsy: 2+. II. Mean of 4 animals. 0·10 I.U. per day. Autopsy: 1+. III. Mean of 2 animals. 0·15 I.U. per day. Autopsy: 1+. IV. Mean of 6 animals. 0·20 I.U. per day. Autopsy: −. V. 1 animal. 0·40 I.U. per day. Autopsy: −.

findings in only 2 animals. A greater degree of comparability is found in the curves corresponding with the doses 0·10, 0·15 and 0·20 I.U., their starting points being rather close together. The smaller the dose, the greater is the initial rise of the curve. But here the maximum is not reached earlier as the dose is increased.

With the greatest dose, 0·40 I.U., the curve does not even show a suggestion of an initial rise, while a small maximum was found in the rats dosed with 10 or 20 mg. cod-liver oil (Fig. 1). This dose—0·40 I.U.—was however only given to 1 animal.

Thus, in tests with cod-liver oil and with irradiated ergosterol, there is a distinct diminution in the initial rise as the dose is increased, even down to complete flattening of the curve.

D. *Young rats on normal diet.*

A litter of young rats was kept on a normal diet—Gudjónsson's diet No. 4 [1930]—and followed in the same manner as the animals above. The autopsy revealed no sign of rickets. The widths of the epiphyseal zones are presented graphically in Fig. 1. Throughout the experiment there was practically no change in the widths of the epiphyseal zones, the curves being almost parallel with the axis of abscissae. The rate of growth was far greater in these animals than in the preceding groups.

Discussion.

From these experiments it is evident that the term "preventive dose" is somewhat ambiguous. The smallest preventive dose—in these experiments, more than 3 mg. cod-liver oil or 0·2 I.U. of the standard vitamin D preparation—was actually a curative dose in so far as the animals showed a distinct condition of radiographic rickets at the beginning of the experimental period, and most pronounced on its 8th day. By increasing the dose a limit was found at which there appeared no sign of rickets whatever throughout the experimental period. Here the doses were 10–20 mg. cod-liver oil or 0·4 I.U. standard vitamin D. The curves for the 3 mg. dose and for the 0·20 I.U. dose are identical, whereas the curves for the largest doses do not show the same degree of conformity. Here it is to be kept in mind, however, that only a few animals had the largest doses of cod-liver oil, and only 1 animal had the largest dose of the standard preparation.

Only the larger doses warrant the employment of the term "preventive dose" in the true sense of the word. I wish to suggest that this dose be called the "real preventive dose." With cod-liver oil it corresponds to the curative dose I have found in previous tests with the curative method of 7 days' duration. The size of this dose is probably dependent upon the vitamin D depots in the young rats when the test commences.

Of course, these experiments demonstrate too that the preventive method allows of comparative estimations only at exactly the same point of time in the experimental period.

It would not be justifiable on the basis of this material to discuss the cause of this peculiar difference between the actions of smaller and larger doses. Two possibilities suggest themselves. (1) That the effect of vitamin D manifests itself only after a certain time, which would explain the initial rise of the curves but not the increasing flattening of the curves with increase in dose. (2) That the rats respond most strongly to the rachitogenic diet in the first part of the experimental period—that the young rat is most susceptible to rickets at this time (age factor?), or that it gradually responds less strikingly to the effects of the diet.

The differences in width of the epiphyseal zones in the various animals when the experiment commences—*i.e.* in 1 month-old rats which have lived under normal conditions—does not appear to be any indication of their differences in susceptibility to rickets. This material demonstrates that the degree of rickets is in no way dependent upon the width of the epiphyseal zone at the commencement of the experiment.

SUMMARY.

1. In negative controls—*i.e.* young rats on McCollum's diet No. 3143 with a daily addition of 0·2 g. peanut oil—the development of rickets progresses with increasing intensity until the 12th day, and then the rachitic changes subside somewhat.

2. Prophylactic addition of vitamin D to this diet inhibits the development of rickets in varying degrees. With the smallest preventive dose there is pronounced radiographic rickets on the 8th day of the experiment, although not so severe as observed in the negative controls.

3. With increasing doses of vitamin D the rachitic changes at the beginning of the experimental period are more and more inconspicuous; and with the real preventive dose there is no sign of rickets whatever.

4. From this it is evident that the preventive method is related to the curative more closely than is generally assumed. The real preventive dose with cod-liver oil is in these experiments equal to the curative dose found by the author in previous experiments. In all the experiments described the breeding stock had been kept on Gudjónsson's diet No. 4, which is rather poor in vitamin D.

REFERENCES.

Bourdillon and Bruce (1932). *Biochem. J.* **26**, 506.
—— —— Fischmann and Webster (1931). *Med. Res. Coun. Lond. Sp. Rep. Ser.*, No. 158.
Gudjónsson (1930). *Biochem. J.* **24**, 1591.
Schultzer (1927). *Biochem. Z.* **188**, 435.
—— (1931). *Biochem. J.* **25**, 1745.

LV. GLUCOSE DEHYDROGENASE: PREPARATION AND SOME PROPERTIES OF THE ENZYME AND ITS COENZYME.

By DOUGLAS CREESE HARRISON.

From the Department of Pharmacology, Sheffield University.

(*Received February 25th, 1933.*)

It was shown recently [Harrison, 1931, 2] that an enzyme, glucose dehydrogenase, which was capable of oxidising glucose, could be extracted from the liver of various animals. It was later found that the oxidation product of glucose was gluconic acid [Harrison, 1932]. Meanwhile, Mann [1932] showed that an activator was concerned in the oxidation of glucose by glucose dehydrogenase. The enzyme preparation which had been described contained both glucose dehydrogenase and the activator or coenzyme. The present paper describes a method by which the dehydrogenase and its coenzyme can be separated and obtained as stable, concentrated preparations containing only traces of pigment and freed from the greater part of the protein which was present in the earlier preparation. The method depends on the fact that the dehydrogenase is precipitated by half saturation of its solution with ammonium sulphate, while the coenzyme is precipitated with the protein which comes down on saturation of the filtrate with ammonium sulphate. Since the coenzyme is moderately thermostable, practically the whole of this protein can then be removed by heat-coagulation.

The material used as a starting-point for the preparation of the enzyme and the coenzyme was acetone liver which was prepared by treating about 1½ lbs. of fresh, minced ox-liver once with slightly more than its own weight of acetone and twice with half its weight of acetone, exactly as previously described [Harrison, 1931, 2], taking the precautions previously mentioned with regard to keeping the temperature low during extraction and removing acetone by prolonged evacuation of the desiccators. To extract the enzyme and coenzyme, 60 g. of the dry acetone liver are put into a mortar with 240 cc. of distilled water and ground up at intervals for 2–3 hours. The mixture is squeezed through muslin, and the residue is ground up with 120 cc. of water and squeezed through muslin, and the combined extracts are centrifuged for a short time. The opaque liquid is poured off (through muslin if a fatty layer is formed) and dialysed overnight in the ice-chest in large (4 cm. × 18 cm.) collodion sacs against 4 litres of distilled water. The collodion sacs are freshly made from an 8 % solution of collodion in equal volumes of alcohol and ether by the method described by Cole [1928]. The solution, the volume of which usually increases by about 50 % during dialysis, is brought to about p_H 5·7 to bromocresol purple by the addition of about 2 drops of *N* HCl and the large protein precipitate is centrifuged off. The clear, or slightly opaque, pigmented solution is made approximately neutral, and an equal volume of saturated ammonium sulphate solution is stirred in.

The precipitate, which contains the glucose dehydrogenase, is filtered off on starch-free pleated filter-papers (Whatman, No. 40), pouring the first part of the filtrate through the paper twice and saving the filtrate for preparation of the coenzyme. The precipitate is blotted, then partially dried on a porous tile. It is then dissolved in 60 cc. of distilled water, centrifuged to remove enzyme-free protein, and reprecipitated by addition of its own volume of saturated ammonium sulphate solution. After filtering, blotting, and partially drying on a porous tile, the precipitate is dried *in vacuo* over sulphuric acid and ground to a homogeneous powder. The weight of the enzyme preparation is usually between 1 and 2 g. from 60 g. of acetone liver, corresponding to about 250 g. of fresh liver. The preparation retains its activity for long periods, especially if stored *in vacuo*. When a solution of the dehydrogenase is required, a suitable weight of the preparation is dissolved in water or buffer solution, brought to the required p_H by cautious addition of *N* NaOH and centrifuged, whereby a further amount of inactive protein is removed. A perfectly clear, pale yellowish-brown solution of glucose dehydrogenase is obtained, which shows practically no reducing power towards methylene blue by itself, and which, in the presence of glucose, reduces methylene blue only slowly unless the coenzyme be added.

The coenzyme is readily prepared by saturating with ammonium sulphate the deeply pigmented filtrate from the first precipitation of the dehydrogenase. The resulting precipitate is filtered on starch-free filter-paper and, after blotting and partially drying on a porous tile, is dissolved in 120 cc. of distilled water and reprecipitated by saturation with ammonium sulphate. The precipitate, after partially drying on a tile, is dissolved in about 30 cc. of distilled water and heated in a boiling water-bath with stirring for 5–6 minutes. The bulky precipitate of coagulated protein is filtered off on a small Büchner funnel and washed with a few cc. of hot water. The combined filtrate and washings are evaporated to dryness either on a water-bath, using a fan to keep the temperature down, or better, in a shallow dish over sulphuric acid in a vacuum desiccator. The dry product, which contains the coenzyme, is ground up to a homogeneous powder. Usually about 0·9 g. is obtained, equivalent to about 250 g. of fresh liver. The preparation usually contains about 90 % of its weight of ammonium sulphate, but this does not interfere with its activity. It keeps well, especially if stored *in vacuo*. On dissolving the requisite amount in water, bringing to the required p_H with *N* NaOH and, if necessary, centrifuging, a perfectly clear, pale yellow solution of the coenzyme is obtained. The solution is almost free from protein and gives only a slight precipitate with sulphosalicylic acid.

The activity of the enzyme with varying amounts of coenzyme is shown in the following experiment. Thunberg vacuum tubes were used, each containing 0·25 cc. 1/5000 methylene blue. The total volume in each tube was made up

Glucose dehydrogenase cc.	Coenzyme cc.	2*M* glucose c.c.	Time in min. for decoloration of methylene blue
0·4	—	—	—
,,	0·08	—	67
,,	0·32	—	26
,,	—	0·2	28
,,	0·02	,,	13·5
,,	0·04	,,	9·25
,,	0·08	,,	5·25
,,	0·16	,,	3
,,	0·32	,,	2·25
,,	0·64	,,	1·75

to 1·55 cc. with distilled water. The dehydrogenase solution (p_H 7·4) contained enzyme equivalent to 0·8 g. acetone liver in each tube, while the coenzyme solution (p_H 7·4) contained the equivalent of 1 g. acetone liver in 0·1 cc. No buffer was used, as the protein in the enzyme solution gave adequate buffering. The tubes were evacuated, filled with nitrogen, re-evacuated and immersed in a water-bath at 37°.

It can be seen that the enzyme and coenzyme together in the absence of glucose bring about slow but relatively negligible reduction of methylene blue. With glucose and the enzyme, the addition of increasing amounts of coenzyme leads to a progressively increasing rate of reduction of methylene blue, the velocity tending towards a maximum with high concentrations of coenzyme. In the above experiment, the maximum velocity of oxidation of glucose in presence of the coenzyme is about 16 times the velocity of oxidation induced by the enzyme alone. Still greater accelerations have sometimes been obtained. In most experiments, amounts of enzyme and coenzyme equivalent to 0·8 g. of the original acetone liver were used, this giving a convenient rate of reduction under the experimental conditions described above.

In another experiment, in an attempt to remove any remaining coenzyme from the enzyme, a portion of the same preparation of dehydrogenase was dissolved in a little water, centrifuged and reprecipitated twice with half saturated ammonium sulphate. On repeating the experiment exactly as before with the practically colourless solution of the purified preparation, only a slight decoloration of the methylene blue had taken place after 6 hours with the enzyme and glucose, while with coenzyme equivalent to 0·8 g. of acetone liver added, the methylene blue was completely reduced in 29 minutes. While considerable loss of enzyme had occurred during purification, this experiment suggests that in the complete absence of the coenzyme, glucose dehydrogenase is incapable of oxidising glucose.

The following are some of the properties of the coenzyme as shown by solutions of the preparation containing ammonium sulphate described above. The coenzyme is only moderately thermostable, and a solution evaporated to dryness several times on the steam-bath, the heating lasting 1·5 hours, lost about 1/3 of its activity. Boiling the solution under reflux for 1 hour destroyed the greater part of its activity. The coenzyme is readily destroyed by warming with dilute acid or alkali. After heating in the steam-bath for 15 minutes in $N/20$ HCl and then neutralising, only a slight activity could be detected, while similar treatment with excess of $N/20$ NaOH brought about complete destruction.

Kaolin at p_H 5 does not adsorb the coenzyme, but it is readily adsorbed from neutral or faintly acid solution by charcoal (Merck's medicinal). Attempted extraction of the washed charcoal with 0·5 % H_2SO_4 was unsuccessful, but subsequent elution with $N/3$ NH_4OH gave a solution with some small activity.

The coenzyme is partially brought down with the $BaSO_4$ precipitate obtained by adding baryta to its solution. On treating such a solution with a slight excess of barium hydroxide, centrifuging and removing the excess of barium with dilute H_2SO_4, the centrifugate showed only about one quarter of its original activity. By treating the washed barium precipitates with excess of 0·5 % H_2SO_4, centrifuging and neutralising, rather more than one quarter of the activity was recovered.

The coenzyme is dialysable through collodion. On dialysing the solution in a small sac against a small volume of distilled water in the ice-chest overnight,

much of the activity was found in the liquid outside the sac. At first sight, it would appear that much of the coenzyme must be lost in the dialysis which is carried out during its preparation. This is not necessarily the case, however, for the coenzyme appears to be strongly adsorbed or combined with the albumin portion of the liver proteins. This is shown by the fact that before removal of these proteins, the coenzyme may be repeatedly precipitated with them by saturation with ammonium sulphate, but that at a later stage, after removal of the proteins by heat coagulation, the coenzyme is not precipitated by saturated ammonium sulphate.

Experiments were carried out to find whether a direct oxidation of glucose by oxygen could be induced by the enzyme-coenzyme system. The experiments were carried out in the Barcroft differential manometer at 37°. It was found, however, that no appreciable increase in oxygen uptake occurred on adding glucose (final concentration 0·25 *M*) to the enzyme and coenzyme solutions even when these were present in amounts equivalent to 6 g. and 12 g. respectively of acetone liver in a volume of 3 cc.

It seemed possible that the coenzyme might be identical with adenylpyrophosphate, the coenzyme of muscle glycolysis. Barium adenylpyrophosphate was accordingly prepared from rabbit muscle by the method of Lohmann [1931]. Estimations of the increase in inorganic phosphorus on heating for 7 minutes in *N* HCl showed that about 60 % of barium adenylpyrophosphate was present in the preparation. Before use, the barium salt was dissolved in the minimum of dilute HCl and freed from barium by adding a slight excess of ammonium sulphate followed by centrifuging and neutralising. On adding the solution to glucose and glucose dehydrogenase, a small acceleration in the reduction of methylene blue was observed, but even with amounts equivalent to 7 mg. of the barium salt in a total volume of 1·55 cc., the effect was very slight compared with that produced by a much smaller concentration of the coenzyme.

The glucose dehydrogenase preparation contains only a small amount of the enzyme which oxidises hexosediphosphate [Harrison, 1931, 1], and this oxidation does not appear to be accelerated by the addition of the glucose coenzyme.

In an attempt to elucidate the mode of action of the coenzyme, an experiment was carried out in which the enzyme and coenzyme were incubated together at 37° for 15 minutes before adding glucose and methylene blue, and the rate of decoloration was compared with that induced by the enzyme with the coenzyme when these had previously been incubated separately for the same length of time. The times of reduction of methylene blue (15·5 and 14·5 minutes respectively) were practically identical, showing that the activity of the coenzyme is not due to its producing an active dehydrogenase from some inactive precursor of the enzyme. Similarly, previous incubation of the coenzyme with glucose did not increase the rate of the reaction on subsequently adding the dehydrogenase. It is clear, therefore, that the coenzyme acts as a direct accelerator of the activation of glucose by glucose dehydrogenase.

The chemical properties of the coenzyme have not been systematically investigated, but it is hoped shortly to publish evidence which has been obtained as to the chemical nature of the active group in glucose dehydrogenase itself.

SUMMARY.

A method is described by which stable, concentrated preparations of glucose dehydrogenase and of its coenzyme may be made from liver. The coenzyme preparation is practically free from protein and both preparations are almost free from pigment.

Some of the properties of the enzyme-coenzyme system are described.

Part of the expense was defrayed by a grant from the Government Grant Committee of the Royal Society.

REFERENCES.

Cole (1928). Practical physiological chemistry, 8th ed., p. 68. (Heffer, Cambridge.)
Harrison (1931, 1). *Biochem. J.* **25**, 1011.
—— (1931, 2). *Biochem. J.* **25**, 1016.
—— (1932). *Biochem. J.* **26**, 1295.
Lohmann (1931). *Biochem. Z.* **233**, 460.
Mann (1932). *Biochem. J.* **26**, 785.

LVI. THE DETERMINATION OF CYSTINE IN BIOLOGICAL MATERIAL.

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(*Received March 1st, 1933.*)

Several methods for the determination of cystine have been put forward, and these fall into three classes.

(*a*) *Iodimetric method.* Okuda [1925] titrated cysteine in the presence of bromide in acid solution with bromate, under which conditions the cysteine is oxidised to cystine and then to cysteic acid. He also titrated cysteine in the presence of iodide in acid solution with iodate, taking the appearance of free iodine as the end-point. In the latter case the oxidation only proceeds as far as cystine. This method could be applied to the estimation of cystine after reduction under suitable conditions. Okuda [1929] modified this procedure by adding to the acid cysteine solution an excess of iodine and titrating back with thiosulphate, the end-point being observed by the decoloration of a chloroform layer of the iodine. Yamazaki [1930] criticised this latter method on the ground that irregular results are obtained by attempting to titrate iodine in chloroform solution and described a potentiometric method using bromate in the presence of bromide or iodate in the presence of iodide. Baernstein [1930] reduced cystine in hydrochloric acid with Devarda's alloy and reoxidised the cysteine with excess iodine in potassium iodide, estimating the excess iodine by measuring the increase in pressure produced by the evolution of nitrogen from hydrazine sulphate in the Van Slyke apparatus.

(*b*) *The colorimetric method of Folin and Marenzi.* This method [Folin and Marenzi, 1929] is a modification of the earlier Folin and Looney [1922] process depending on the production of a colour with the Folin-Looney uric acid reagent and cysteine, cystine being reduced with alkaline sulphite. Lugg [1932, 2] has described a modification of this method in which corrections are applied for the presence of extraneous reducers in the solution.

(*c*) *The Sullivan colorimetric method.* Sullivan [1926] introduced a test for cystine based on the colour produced with sodium-β-naphthoquinonesulphonate and cysteine, and developed this into a quantitative method of estimation. The reaction takes place in alkaline sulphite solution and is followed by an addition of sodium hydrosulphite, under which conditions the reagent is specific for cysteine. A large number of biologically important substances were tested and with the exception of cystine were found to give no colour with the reagent under these conditions.

All these methods with the exception of that of Baernstein [1930] have the disadvantage that protein hydrolysates must be treated with decolorising material, during which process an inevitable loss of cystine is sustained. Folin and Marenzi [1929] substituted kaolin for the charcoal previously employed by Folin and Looney [1922] and claimed that the loss of cystine was reduced. As regards the iodimetric methods Baernstein [1930] claimed that under the con-

ditions of his method iodine is specific for the sulphydryl group of cysteine, while later [1932] he stated that the method was incapable of distinguishing cysteine from other sulphydryl compounds. The latter statement is borne out by the fact that his results are much higher than those of other workers. Yamazaki [1930] states that iodate in the presence of iodide is reduced by tryptophan. Other disadvantages of the methods of Okuda [1925] and Yamazaki [1930] are that the results depend on the temperature and the speed with which the titration is carried out. Lucas and King [1932] have investigated the reaction between cysteine and iodine and have shown the effects of temperature, acidity and the presence or absence of iodide on the iodine titre of cysteine to be considerable.

The Folin and Marenzi method is open to criticism on various grounds. The sulphite treatment only gives 50 % reduction of cystine. Mirsky and Anson [1930] have pointed out that equivalent quantities of cysteine and cystine give colour intensities in the ratio of 1·75 : 1, while Vickery and White [quoted by Clarke, 1932] find a ratio of 2 : 1. In addition to this is the fact that the colour fades rapidly on exposure to the air, so that determinations must be carried out under carefully standardised conditions to obtain agreement. Pirie [1932] has questioned the specificity of the reaction as it would appear that methionine present in caseinogen that has been treated with nitrous acid reacts with the uric acid reagent. Lugg [1932, 1] has studied the effect of the conditions on the reduction of the uric acid reagent by cysteine and has also shown that other substances will reduce the reagent to a varying degree.

The chief disadvantage of the Sullivan [1926] reaction is the formation of double decomposition products between cystine and cyanide, and cystine and sulphite, in alkaline solution [Clarke, 1932]. Sullivan has pointed out that the degree of correspondence given between cystine and cysteine solutions varies with the concentration and that reduction of cystine only indicates 50 to 75 % of the theoretical compared with cysteine.

Experimental.

An attempt has been made to overcome the difficulties of the Sullivan reaction by starting with preformed cysteine in the following way.

Hydrolysis of the protein. An amount of the material to be tested, containing approximately 10 to 20 mg. of cystine, was hydrolysed by boiling with 45 cc. of 20 % hydrochloric acid for 6 to 8 hours. On cooling, the solution was partially neutralised with approximately 23 cc. of 40 % sodium hydroxide, cooled, filtered and finally adjusted to p_H 2 by the further addition of 2 to 3 cc. of the sodium hydroxide, using thymol blue as indicator. The volume was made up 100 cc. with $N/10$ hydrochloric acid using washings from the original filter.

Determination. The following solutions were employed:

25 % sodium hydroxide,
5 % potassium cyanide,
0·5 % sodium β-naphthoquinonesulphonate,
10 % sodium sulphite in $N/2$ sodium hydroxide,
2 % sodium hydrosulphite in $N/2$ sodium hydroxide.

The reagents must be freshly made up and the sodium β-naphthoquinonesulphonate must be made up each day.

5 cc. of the hydrolysate were reduced with a few mg. of zinc dust by heating for 5 minutes in the water-bath. The suspension was filtered into a

flask containing a mixture of 4 cc. potassium cyanide and 1 cc. of sodium hydroxide, giving an almost colourless filtrate. The vessels and filter were washed with 3 cc. of hot water and then 2 cc. of the sodium β-naphthoquinonesulphonate and 5 cc. of sodium sulphite added. After standing for 30 minutes in stoppered vessels 1 cc. of sodium hydrosulphite was added, and the colour compared in the colorimeter with that produced by 5 cc. of standard cystine solution treated in the same way[1]. Convenient standards are 10, 15 and 20 mg. cystine per 100 cc. in $N/10$ hydrochloric acid.

This procedure overcomes the disadvantages of the original Sullivan [1926] method by introducing the cystine into the alkaline sulphite-cyanide mixture as preformed cysteine, and by making use of the zinc reduction in acid solution as a decolorising agent in place of charcoal adsorption. The zinc is not precipitated at any stage but remains in solution as the complex cyanide. Polarimetric observation showed that the reduction of *l*-cystine with zinc and hydrochloric acid as employed in the estimation was complete within 2 to 3 minutes. It is important to carry out the determination on the fresh hydrolysate as low results are obtained if this precaution is neglected. This may be due to atmospheric oxidation of the cystine in acid solution.

Results.

Table I shows some results obtained by use of this method compared with those of other workers. There is good agreement with the Folin and Marenzi [1929] process. The higher value for egg-white is accounted for by the presence

Table I. *The cystine contents of various proteins obtained by this method and compared with those obtained by other investigators.*

	Cystine % Modified Sullivan method				
	Hydrolysis				
Material	1	2	Mean	Cystine %	Other investigators
B.D.H. light white caseinogen 1	0·33	0·31	0·32	1·2–2·5	Sherman and Woods [1925] (Biol. method)
2	0·31	0·32		0·30	Folin and Marenzi [1929]
				0·3	Sullivan [1929]
Prideaux caseinogen	0·32	0·32		0·94	Baernstein [1930]
				0·65	Baernstein [1932]
Hammarsten caseinogen	0·21	0·23	0·22	0·22	Folin and Marenzi [1929]
Dried egg-white	1·48	1·43	1·45	1·22	(Purified egg-albumin) Folin and Marenzi [1929]
				2·07	Baernstein [1930]

of proteins richer in cystine than egg-albumin. The iodimetric method gives much higher results. From the work of Pirie [1932] on the isolation of methionine it is probable that a value of 0·3 % cystine for caseinogen is close to the actual.

In addition the following determinations of cystine were made.

Purified caseinogen. The caseinogen was purified in this laboratory by dissolving B.D.H. "light white casein" in hot tap-water to give a 5 % solution and precipitating by the addition of acetic acid. The caseinogen was washed by decantation with 0·5 % acetic acid, extracted in a Soxhlet apparatus for 96 hours

[1] After this paper had gone to press it was found that gelatin hydrolysates, in contrast to the other hydrolysates tested, gave a colour which rapidly faded.

with 70 % alcohol containing 0·03 % acetic acid and then washed with 93 % alcohol. After drying in an air-oven at a low temperature it was ground and heated for 3 days at 120° [Chick and Roscoe, 1928]. The cystine content of this product was found to be 0·25, 0·23, 0·24 %. Manufacturers' products were compared with this: B.D.H. fat-free and vitamin-free caseinogen 0·21, 0·20 % cystine; "Casein-Harris" 0·23, 0·23 %; Glaxo caseinogen (physiological caseinogen AB) 0·11, 0·11 %. The caseinogen purified in this laboratory agrees closely in its cystine content with that of Hammarsten caseinogen (0·22 % cystine) and it was found that no loss of cystine was sustained in the heating process to which it was subjected.

Whole milk powder. This was prepared from winter milk by the roller process and gave on analysis 0·17, 0·17 % cystine.

Dried yeast. The yeast was dried for 3 days at 37°. One sample of brewer's top yeast gave 0·92, 0·92 % cystine; another sample 0·52, 0·51 %. A sample of yeast from Pharmaco-Chemical Products, Ltd., gave 0·67, 0·62 % cystine.

Cereals. Whole S. American yellow maize 0·15, 0·17 %; whole soft red English wheat 0·31, 0·30 % cystine.

Summary.

1. A modification of the Sullivan [1926] method for determining cystine is described in which the cystine is first reduced with zinc in acid solution.

2. Estimations of cystine in various substances were carried out. Some of these are compared with results obtained by other workers and show good agreement with those of Folin and Marenzi [1929].

My thanks are due to the Lister Institute for hospitality, and to Miss M. H. Roscoe, Miss E. M. Hume, Mr N. W. Pirie and Prof. R. Robison for advice.

References.

Baernstein (1930). *J. Biol. Chem.* **89**, 125.
—— (1932). *J. Biol. Chem.* **97**, 669.
Chick and Roscoe (1928). *Biochem. J.* **22**, 790.
Clarke (1932). *J. Biol. Chem.* **97**, 235.
Folin and Looney (1922). *J. Biol. Chem.* **51**, 427.
—— and Marenzi (1929). *J. Biol. Chem.* **83**, 103.
Lucas and King (1932). *Biochem. J.* **26**, 2076.
Lugg (1932, 1). *Biochem. J.* **26**, 2144.
—— (1932, 2). *Biochem. J.* **26**, 2160.
Mirsky and Anson (1930). *Proc. Soc. Exp. Biol. Med.* **28**, 170.
Okuda (1925). *J. Biochem.* (Tokio), **5**, 217.
—— (1929). *Nihon Nogeikagakukai Zasshi*, **5**, 549.
Pirie (1932). *Biochem. J.* **26**, 1271.
Sherman and Woods (1925). *J. Biol. Chem.* **66**, 29.
Sullivan (1926). *U.S. Pub. Health Rep.* **41**, 1030.
—— (1929). *U.S. Pub. Health Rep. Supp.* No. 78.
Yamazaki (1930). *J. Biochem.* (Tokio), **12**, 207.

LVII. METABOLISM OF TISSUES GROWING IN CULTURE.

V. EFFECT OF RADIUM ON THE METABOLISM OF CULTURES OF EMBRYONIC KIDNEY TISSUE.

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(*Received March 1st, 1933.*)

In previous papers we have described our technique for estimating the nitrogen and the sugar metabolism of rat-kidney tissue in culture, and also the effect of various sugars in preventing the formation of urea and ammonia by the cultures [Holmes and Watchorn, 1927; Watchorn and Holmes, 1927; 1931]. In recapitulation here it is only necessary to say that "growing" tissues show a formation of urea and ammonia which is not found in tissues which are not growing, that the presence of glucose and fructose in the medium always and that of galactose sometimes prevents the formation of urea and ammonia (although accelerating the growth), while xylose has no such effect. Glucose, fructose, galactose and xylose are all broken down by the tissue, as shown by estimation of reducing substances at the beginning and end of the culture period. Later work has shown that glucose is the most extensively used and that xylose breakdown is sometimes very small.

In addition to this, recent work has brought out one new and interesting point. The increase in area of the cultures of kidney tissue, which we have always spoken of as "growth" of the cultures, we had formerly considered to be due to cell division as well as cell out-wandering. Lately, however, attempts at counting mitotic figures have shown that no division is taking place. It is possible that cell division continues for a few hours after the planting of the culture, but there is very little doubt that by far the greater part of the increase in area is due to the wandering of cells away from the fragment planted. It is therefore interesting to note that, in the absence of suitable carbohydrate, cell movement is apparently supported by the breakdown of nitrogen-containing substances. A recent paper by Clark *et al.* [1931] has shown that the beating heart oxidises protein as well as carbohydrate and produces urea and ammonia, and it seems likely that the production of urea and ammonia by the wandering cells is due to a similar process. In the absence of out-wandering these substances are not produced by the culture. Clark, however, finds that protein and carbohydrate are oxidised at the same time, whereas in these cultures the presence of carbohydrate in the ordinary physiological concentrations inhibits the protein breakdown.

Experiments with radium.

The effect of radium upon tissue cultures is well known but puzzling, and very little is known which might help to explain it. It was felt that a knowledge of the metabolism of irradiated cultures might be helpful. The source of

irradiation used was a 300 mg. plaque, the radium container being of platinum 0·5 mm. in thickness, this ensuring that only γ-rays reach the tissues. The tissue was planted as usual on strands of cotton-wool in a pyrex flask containing a little embryo extract, and was arranged so that as far as possible all the tissue should lie immediately over the radium container, which was 0·5 cm. distant from the bottom of the flask.

A very small dose of γ-radiation is known to cause a temporary stoppage of cell division, but a very large dose is needed to cause the death of the cells in culture. Spear has shown [1930] that an enormous dose of γ-rays will prevent immediately any out-wandering from a culture, which is then spoken of as dead, and that rather smaller doses will cause death after a latent period, the latent period becoming longer as the doses become smaller. In cultures in which delayed death is going to occur, the appearance of the cells shortly after irradiation is fairly normal and occasionally mitoses are seen, but these soon cease and the cells appear progressively more abnormal. Out-wandering, however, may continue for several days or even weeks until eventually the culture dies. The actual cause of death is unknown, but many suggestions have been made, among others, that slow self-poisoning of the cells may be taking place owing to an abnormal metabolism.

The duration of exposure to radium in the experiments quoted below was 14 hours. Shorter exposures were first tried, but although similar results were obtained they were irregular and therefore unsuitable for work of this kind, in which the results of one experiment have to be compared with the results of another experiment carried out at another time.

Supposing that the kidney tissue was equal in sensitivity to the fibroblasts used by Spear, the death of the cultures would not occur for about 10 days, whereas these cultures were taken for estimation after 2 days.

With an exposure of 14 hours it was regularly found that the breakdown of both glucose and galactose was cut down, usually to about 50 or 60 % of the breakdown found in the control. Table I gives typical examples.

Table I. *Mg. sugar present after 2 days.*

Original amount	Non-growing tissue	Control growing tissue	Irradiated growing tissue	Percentage inhibition of sugar breakdown
		Glucose		
1·91	1·29	1·1	1·50	50
2·26	1·82	1·40	1·68	33 (only part of the tissue could be irradiated)
		Galactose		
1·31	1·03	1·03	1·15	43
1·31	—	0·95	1·11	44

As one of the theories of the action of γ- and X-rays on tissues postulates the damage of the colloid structure of the cell, we thought it might be worth while to injure the cell structure by freezing below $-7°$ and compare the effect on carbohydrate breakdown of this freezing with that obtained after radium. We accordingly froze one culture containing glucose to $-12°$, which should cause the formation of ice-crystals inside the cells and injure the structure by so doing. In this case, whereas 0·63 mg. of glucose was broken down by the control, only 0·12 mg. was broken down by the frozen culture. This is a similar effect to that produced by radium, though more extreme.

The effects of radium on protein metabolism had to be tested in cultures to which no glucose or galactose had been added, since these sugars themselves prevent protein breakdown. This is unfortunate, as it has been suggested by some authors [Frik and Posener, 1926; Mayer, 1926] that *in vivo* the injection of large amounts of glucose can sensitise the tissues (tumour) of an animal to irradiation. However, the sugar contents of the glucose- and galactose-containing cultures were rather lower than the ordinary physiological level of the body fluids, so that any effect of excessive amounts of sugar would not come into consideration. Moreover it was possible to add xylose as a control in the nitrogen experiments, as this sugar had been found to have no effect on protein metabolism, and in most cases xylose was present in the experiments given in Table II.

Table II. *Mg. NH_3- and urea-nitrogen.*

Non-growing	Control growing	Increase	Irradiated growing	Increase	Remarks
0·127	0·127	0	0·142	0·015	—
0·114	0·144	0·030	0·175	0·061	4 kidneys; more tissue than usual
0·090	0·126	0·036	0·109	0·019	Known to have been badly planted
0·093	0·118	0·025	0·118	0·025	—
0·070	0·106	0·036	0·093	0·023	—

There is no doubt that under the conditions of these experiments the protein breakdown was far less easily affected by irradiation than the carbohydrate breakdown. The figures given show that the radium had probably no effect at all, and that only the ordinary variation due to varying extents of out-wandering in the cultures was recorded. At most, there is an occasional, irregular inhibition of protein breakdown such as could be produced with much smaller doses of γ-radiation in the case of carbohydrate breakdown. The protein metabolism is estimated by the increase in ammonia- and urea-N in the "growing" cultures, as compared with cultures which could not "grow" because the fragments were floating freely in the medium (see earlier papers).

It will be seen that the irradiated tissues twice gave a higher answer, once the same answer, and twice a lower answer. Moreover, one of these low answers could be fully accounted for by bad planting of the tissue.

Several experiments show that no autolytic formation of urea and ammonia takes place in cultures after irradiation. In the following experiments growth was for various reasons not found in the cultures, and in these the irradiated cultures, like the controls, show no ammonia or urea formation (Table III).

Table III. *Mg. NH_3- and urea-nitrogen.*

Non-growing	Control "growing"	Irradiated "growing"
0·090	0·095	0·092
0·097	0·094	0·091
0·088	0·088	0·088
Non-growing	Control "growing"	Frozen "growing"
0·089	0·108	0·085

The non-formation of autolytic urea and ammonia does not prove that injury to the structure of the cell has not taken place, since freezing to $-12°$, which almost certainly injures the structure, entirely prevents growth (Table III) but does not cause the appearance of autolytic ammonia and urea during the 2 days experiment. Obviously, however, any injury due to the radium must

be far less gross than any due to the freezing, since the radium does not prevent a normal protein breakdown in wandering cells. The apparently selective action of radium does not support the theory that its effect is merely one of damage to colloidal structure, since this affects both types of metabolism equally.

Further support is given to the idea that the protein metabolism is very insensitive to the action of γ-rays by the fact that microscopical examination of stained cultures shows that out-wandering is just as extensive after irradiation as it is in the controls. We have always considered that the amount of protein breakdown (in the absence of carbohydrates) is determined by the amount of out-wandering or "outgrowth," so that the microscopical findings confirm the chemical, and show that no depression of protein metabolism is likely to be found during the first 2 days after the 14 hours' exposure to γ-rays.

Acceleration of carbohydrate breakdown.

In earlier experiments, when lower intensities of irradiation were being used, quite definite acceleration of galactose breakdown was obtained on two occasions out of four or five experiments. There is enough variation in the sensitivity of cultures from one experiment to another to make it difficult to repeat a result of this sort, and more time could not be spent in the attempt.

Unfortunately the exact intensity of the irradiation received by the tissues in these particular experiments is difficult to determine, but it was certainly very much less than that required to decrease sugar breakdown or to bring about death without a very long latent period occurring after the exposure to radium.

Increases of metabolism after irradiation have been reported by several observers, but it is usually thought that these represent responses to slight injury rather than real stimulation of the cells. It is difficult to picture any type of injury to these cultures which could produce an increase in carbohydrate breakdown.

Table IV. *Mg. of galactose disappearing.*

Non-growing		Growing	
Control	Irradiated	Control	Irradiated
0·36	0·54	—	—
0·21	0·37	0·25	0·43
—	—	0·35	0·52

Table IV gives the figures for the two experiments mentioned, showing the increased disappearance of galactose under the influence of radiation.

Discussion.

A search of the literature shows that the facts already known about the effects of radiation on cell metabolism are contradictory and difficult to reconcile with one another. Crabtree [1932], using surviving tissues in a glucose-bicarbonate Ringer's solution, found that the respiration of the tissues was very much reduced by radiation long before the glycolysis was affected. Krontowski [1933] also found that, with tissue cultures suffering a delayed death after irradiation, the glycolysis continued almost unaltered at the end of 10 days, although all out-wandering had ceased; considerably higher doses of radium were needed to produce an alteration in glycolysis than to produce delayed death. Apart from any question of the effect of the radiation, Krontowski's

results are very difficult to understand, since we have found that actively wandering cultures have a higher carbohydrate metabolism than cultures that are not out-wandering. This is surely the result that would be expected, and, if true for tissues which though not showing out-wandering are perfectly healthy, is still more likely to be true of cultures which have ceased their cell movement owing to the delayed injurious effect of irradiation.

Frik and Posener [1926], on the other hand, found that, *in vitro*, glycolysis was far more easily affected by X-rays than was the respiration of the tissues. The most striking example of this was given by the retina, which proved to be very sensitive to radiation, and in which glycolysis could be completely inhibited by exposures which left the respiration unaltered. Wels [1924] irradiated yeast and other cells and found the respiration rather insensitive; exposures which affected the viable count of staphylococcus cultures did not cut down the respiration.

The results recorded in this paper would presumably agree with those of Frik and Posener, since glycolysis is stopped but protein oxidation continues and could take the place of carbohydrate oxidation.

It is obviously difficult to reconcile these conflicting results with one another, but there are possible explanations of the differences found.

Crabtree and Krontowski both used radium containers which would allow of the passage of a certain amount of β-radiation. Though the proportion of β- to γ-radiation was very small, the influence of β-radiation on biological material is so very great that its effects would have been apparent. It is not at all certain that the effects of β-radiation on metabolism would be the same as the effects of γ-radiation and X-rays. In Crabtree's interesting experiments the tissues were surviving but not growing in culture, and it is possible that the striking recovery of cells from the immediate effects of γ-rays, which is found in tissue cultures, might not be present in his preparations. In this case no doubt different answers would be given by tissue culture and by surviving tissue experiments.

A further clue to the reason for the many discrepancies is given by the work of Frik and Posener already quoted. These authors found that although total glycolysis is much cut down and respiration unaltered, aerobic glycolysis may actually be greater in the irradiated tissues; in other words that although oxidation continues, it is not oxidation of the products of carbohydrate breakdown. For some tissues under some conditions the cutting down of carbohydrate oxidation would mean a large decrease in the whole oxygen uptake, whereas under other conditions and with provision of suitable substrates, oxidation of protein and other substances might take the place of carbohydrate oxidation. Tissues in culture are provided with plenty of utilisable protein substances, and kidney tissue in particular probably oxidises substances other than carbohydrate quite readily.

The different components of the rather complex enzyme system by which glycolysis is brought about in cells are known to vary quantitatively even if not qualitatively from one tissue to another. This is likely to make the glycolytic systems of some tissues more sensitive to radium than those of other tissues. Comparison of the effects of radium on different tissues as well as a study of its action on as many enzyme systems as possible should show whether there is really any selective action, and if so what is the most vulnerable part of the cell organisation.

SUMMARY.

1. In the absence of suitable carbohydrate, cell movement appears to take place at the expense of protein breakdown.

2. Fourteen hours' exposure at a distance of 0·5 cm. to the γ-rays from 300 mg. radium produces a 40 or 50 % inhibition of carbohydrate breakdown. Smaller doses (*e.g.* 9 hours) produce this effect only irregularly, and small doses may actually produce an acceleration of breakdown.

3. Fourteen hours' exposure produces no effect or at most a very irregular effect on protein breakdown, which therefore appears to be much less sensitive to γ-radiation.

4. Fourteen hours' exposure on several occasions produced no visible effect on the increase in area of the culture.

5. The possible significance of the contradictory results obtained by several workers in this field is discussed.

A great deal of this work was carried out with the co-operation of Miss E. Watchorn, who was only prevented by lack of time from continuing it.

My most grateful thanks are due to Dr Spear for his continued advice and help, without which the work would not have been possible.

I have to thank the Medical Research Council for a personal grant.

REFERENCES.

Clark, Gaddie and Stewart (1931). *J. Physiol.* **72**, 443.
Crabtree (1932). 10th Sci. Ref. Imp. Cancer Res. Fund, p. 33.
Frik and Posener (1926). *Fortschr. Gebiet Röntgenstrahlen*, **34**, 46. (Kongressheft.)
Holmes and Watchorn (1927). *Biochem. J.* **21**, 327.
Krontowski (1933). *Arch. exp. Zellforsch.* **13**, 407.
Mayer (1926). *Fortschr. Gebiet Röntgenstrahlen*, **34**, 37. (Kongressheft.)
Spear (1930). *Proc. Roy. Soc. Lond.* B **106**, 44.
Watchorn and Holmes (1927). *Biochem. J.* **21**, 1391.
—— —— (1931). *Biochem. J.* **25**, 843.
Wels (1924). *Pflüger's Arch.* **203**, 262.

LVIII. THE REACTION BETWEEN NAPHTHALENE-2-SULPHONIC CHLORIDE AND SOME THIOL COMPOUNDS.

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(*Received March 7th, 1933.*)

FISCHER AND BERGELL [1902] described a highly efficient method for the identification of amino-acids and polypeptides by means of their *N*-naphthalene-2-sulphonyl derivatives. These compounds are generally highly crystalline, soluble in alkalis, insoluble in acids and, since they contain sulphur, introduce an additional factor into their analytical determinations. Hopkins [1929] described unsuccessful attempts to prepare the naphthalene-2-sulphonyl derivative of glutathione, a fact which at first sight seems puzzling in view of the facile preparation of corresponding derivatives from other amino-acids and peptides. No explanation of the fact was there given. The matter has been re-investigated and considerable light has been thrown upon the course of the reaction.

Pure crystalline glutathione prepared according to the method of Hopkins as modified by Pirie [1930] was dissolved in *N* sodium hydroxide (3 mols.) and shaken with an ethereal solution of naphthalene-2-sulphonic chloride. The ethereal layer was separated and the alkaline aqueous layer acidified with dilute hydrochloric acid. A white crystalline precipitate was produced which contained no nitrogen but had a high sulphur content and proved to be naphthalene-2-sulphinic acid in a very pure condition. This at once suggested that the glutathione had reduced the naphthalenesulphonic chloride to sodium naphthalenesulphinate, GSH at the same time being oxidised, presumably to GSSG. To test this speculation GSSG was treated with naphthalenesulphonic chloride as above. Here again, however, naphthalenesulphinic acid was obtained in a remarkably pure condition, but in reduced yield.

This being so the reactions were re-investigated quantitatively. Under the conditions cited in the experimental part, it was found that 1 mol. of GSH gave rise to $1\frac{1}{2}$ mols. of sulphinic acid. The ethereal layer was found to contain nothing but 0·5 mol. unchanged naphthalenesulphonic chloride. The acid filtrate from the sulphinic acid was shaken with ether but nothing (except traces of sulphinic acid in some cases) was extracted, showing that no ether-soluble substances had been formed as the result of the reaction.

Since 2 mols. of sulphonic chloride were employed, of which $1\frac{1}{2}$ mols. disappeared with the formation of $1\frac{1}{2}$ mols. of sulphinic acid, it is clear that the function of the sulphonic chloride is purely that of an oxidising agent and these quantities point to the probable formation of a disulphoxide of glutathione

which one would expect to be very water-soluble. The following equations express this change:

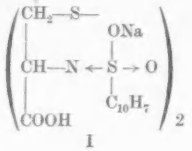
$$2GSH + C_{10}H_7SO_2Cl + 2NaOH = GSSG + C_{10}H_7SO_2Na + NaCl + 2H_2O$$
$$GSSG + 2C_{10}H_7SO_2Cl + 4NaOH = GSO_2SG + 2C_{10}H_7SO_2Na + 2NaCl + 2H_2O^1$$

It seems somewhat strange that the excess of sulphonic chloride does not react with the amino-group of glutathione. It may be that the *N*-sulphonyl derivative is momentarily formed to some extent, but that the reducing action of glutathione or perhaps of the accumulating sulphinic acid is sufficient to regenerate the peptide although this is unlikely (*cf.* reduction of sulphonyl derivatives of amino-acids [Fischer, 1915]).

The yield of sulphinic acid from GSSG also pointed to the formation of a disulphoxide. The oxidised product, whatever its nature, must for reasons stated above be in the filtrate after the separation of sulphinic acid and since nothing could be extracted from it with non-polar solvents it was evaporated to dryness in a desiccator giving a mixture of sodium chloride and a white deliquescent substance. The latter was extracted with absolute alcohol giving a solid on being dried over P_2O_5 in a desiccator. This was certainly not a pure substance, but probably contained the disulphoxide. It gave low N and S figures and has so far resisted further efforts at purification. It readily reduced aqueous potassium permanganate in the cold, also bromine, iodine, mercuric chloride and ammoniacal silver nitrate. It evolved ammonia when boiled with caustic soda, and gave the nitroprusside test after reduction with zinc and dilute sulphuric acid in the cold.

The reaction between GSH and GSSG and naphthalenesulphonic chloride was carried out in the absence of air as far as possible in order to determine whether molecular oxygen played any part. The results however were identical with the above.

Attention was then turned to the reaction between naphthalene-2-sulphonic chloride and cysteine. Abderhalden and Wybert [1916] have already shown that *l*-cystine reacts with two molecules of the sulphonic chloride giving di-naphthalene-2-sulphonyl-*l*-cystine. Their work was repeated and it was found that on very careful acidification of the alkaline layer a crystalline precipitate was produced at about p_H 8·8. This was isolated and was undoubtedly a sodium salt probably having the constitution I.



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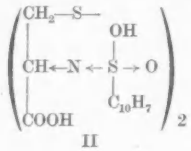

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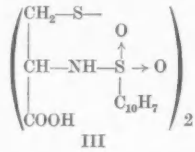

III

It is a stable compound and can be recrystallised from water or alcohol. On treatment with dilute hydrochloric acid in the cold it gave an oily subtance

[1] If a disulphoxide of glutathione does exist it will most probably have the formula and therefore be a thiolsulphonate (*cf.* Smiles and Gibson [1924] and Miller and Smiles [1925]). This point cannot be decided until the substance is obtained pure; but the fact that the crude product gives a nitroprusside test after being gently warmed with NaOH points to this:

$$GSO_2SG + H_2O = GSO_2H + GSH.$$

which crystallised only on long standing. On the other hand if the sodium derivative or the oil were boiled with dilute hydrochloric acid, a crystalline product was immediately obtained, which proved to be identical with that obtained by Abderhalden (III). The oil is in all probability the metastable tautomeric form II.

The separation of the sodium salt at p_H 8·8 was not noted by Abderhalden and Wybert, but this proved of value in isolating the products from the reaction between cysteine and naphthalenesulphonic chloride. On acidifying the alkaline layer after the reaction a gum was obtained from which nothing could be isolated, but on careful acidification to p_H 8·8 the sodium derivative was separated. The filtrate was then strongly acidified with dilute hydrochloric acid, whereupon a precipitate of naphthalene-2-sulphinic acid was obtained, the yield corresponding with the change —SH to —SS—.

The reaction then is given by the following equation:

$$2\begin{matrix}CH_2SH\\|\\CH.NH_2\\|\\COOH\end{matrix} + 3C_{10}H_7SO_2Cl + 6NaOH = \left(\begin{matrix}CH_2—S—\\|\\CH.NNaSO_2C_{10}H_7\\|\\COOH\end{matrix}\right)_2 + 3NaCl + C_{10}H_7SO_2Na + 6H_2O$$

In order further to substantiate the fact that the appearance of naphthalene-2-sulphinic acid is due to the reducing effect of the —SH group in cysteine, the latter was converted into *S*-benzylcysteine and then treated with naphthalenesulphonic chloride. An almost quantitative yield of *S*-benzyl-*N*-naphthalene-2-sulphonylcysteine was obtained and no trace of sulphinic acid was found. This compound (IV) does not appear to have been described hitherto.

$$\begin{matrix}CH_2S.CH_2.C_6H_5\\|\\CH.NH.SO_2C_{10}H_7\\|\\COOH\end{matrix} \qquad \text{(IV)}$$

Attempts were made similarly to prepare *S*-benzylglutathione and determine its behaviour towards naphthalenesulphonic chloride. The reaction between glutathione and benzyl chloride is, however, abnormal[1].

A much simpler case was then investigated, namely the reaction between thiolacetic acid and naphthalenesulphonic chloride. The only products were dithioglycollic acid and naphthalene-2-sulphinic acid, the yield of the latter being in accordance with the change —SH to —SS—. This falls into line with the observation of Otto [1891] who showed that benzenesulphonic chloride reacted with thiophenol giving benzenesulphinic acid.

Experimental.

Reaction between naphthalene-2-sulphonic chloride and glutathione.

1·23 g. of glutathione were dissolved in 8 cc. *N* NaOH and added to a solution of 1·81 g. of naphthalene-2-sulphonic chloride dissolved in ether. The mixture was shaken mechanically, three further quantities of 4 cc. of *N* NaOH being added at hourly intervals. The shaking was continued for 1 hour after the final addition. At the end of 4 hours the ethereal layer was separated and dried over calcium chloride; most of the ether was distilled off and the residue

[1] This reaction and also the reaction between glutathione and 2:4-dinitrochlorobenzene are at present being investigated and the results will be communicated in a later paper.

allowed to crystallise in a weighed evaporating dish; weight of dried unchanged naphthalenesulphonic chloride 0·39 g. M.P. 79°. The aqueous layer was acidified with dilute hydrochloric acid and allowed to stand for a short time to ensure complete precipitation. The naphthalenesulphinic acid was filtered off, washed with water and dried: yield 1·1 g.; M.P. 105°. Found S = 16·5 %, $C_{10}H_8O_2S$ requires S = 16·67 %. With ferric chloride the acid formed the pale yellow crystalline ferric salt insoluble in dilute mineral acids as described by Thomas [1909].

The following table shows the theoretical quantities required to bring about oxidation to the disulphoxide compared with the quantities actually found in this and two similar subsequent experiments.

	GSH used g.	Sulphonic chloride taken g.	Sulphonic chloride recovered g.	Sulphonic chloride used up g.	Yield of sulphinic acid g.
Theoretical	1·23	1·81	0·45	1·36	1·15
Exp. 1	1·23	1·81	0·39	1·42	1·10
„ 2	1·23	1·81	0·36	1·45	1·05
„ 3	1·23	1·81	0·38	1·43	1·10

The filtrate from the sulphinic acid had strongly reducing properties. It reduced potassium permanganate and bromine water rapidly in the cold, iodine somewhat less rapidly. It also reduced ammoniacal silver nitrate and mercuric chloride. The solution was evaporated to dryness in a desiccator and extracted with absolute alcohol which on evaporation over P_2O_5 in a desiccator gave a deliquescent solid. This was obviously not a pure substance and probably still contained some alcohol. Found N = 7·86 %, S = 7·74 %. (The disulphoxide would require N = 13·05 %, S = 9·91 %.) It had however all the reducing properties of the original solution and after reduction with zinc and dilute sulphuric acid gave the nitroprusside test. On being warmed with NaOH ammonia was given off, the residue giving the nitroprusside test.

Reaction between naphthalene-2-sulphonic chloride and GSSG.

Oxidised glutathione was prepared according to the method of Pirie [1931]. The reaction was carried out as for GSH; on acidification of the alkaline layer sulphinic acid was precipitated: yield = 0·35 g. from 0·612 g. GSSG. (Amount theoretically required for disulphoxide formation = 0·38 g.) Found S = 16·51 %. M.P. 105°.

The above experiments were also carried out as far as possible in the absence of molecular oxygen. The sulphonic chloride was dissolved in toluene and added to the alkaline solution of GSH or GSSG in a stout round-bottomed flask, the latter being evacuated until the toluene began to boil. It was then shaken as before. The percentage yields of sulphinic acid were similar to those obtained in the presence of molecular oxygen.

Dinaphthalene-2-sulphonyl-l-*cystine* (*cf.* Abderhalden and Wybert [1916]).

1·6 g. of cystine were dissolved in 14 cc. *N* NaOH and shaken with 6·04 g. of naphthalene-2-sulphonic chloride dissolved in ether. At hourly intervals 14 cc. *N* NaOH were added, three such additions being made. At the end of 4 hours the ethereal layer was separated and dilute hydrochloric acid added gradually to the alkaline layer until still just alkaline to cresol red. The solution was scratched and crystallisation set in rapidly. The solid was filtered off and crystallised from aqueous alcohol in short white needles. It foams and blackens

at 214°. Found N = 3·9 %, Na[1] = 6·64 %, $C_{26}H_{22}O_8N_2S_4Na_2$ requires N = 4·22 %, Na = 6·93 %.

This sodium derivative was dissolved in water and dilute hydrochloric acid added; an oily substance was produced in the cold, but if boiled white needles were produced. These were filtered off, washed with water and recrystallised from alcohol: yield, theoretical on sodium salt taken: M.P. 216°. Found S = 20·4 %, N = 4·3 %, $C_{26}H_{24}O_8N_2S_4$ requires S = 20·67 %, N = 4·52 %.

Reaction between cysteine and naphthalene-2-sulphonic chloride.

3·15 g. of cysteine hydrochloride were dissolved in water and converted into the free amino-acid by adding the calculated quantity of NaOH. 20 cc. of *N* NaOH were then added and shaken with a solution of 9·06 g. of sulphonic chloride in ether. Three further quantities of 20 cc. of NaOH were added at hourly intervals. At the end of 4 hours the alkaline layer was acidified with dilute hydrochloric acid to p_H 8·8, whereupon the sodium salt separated. This was recrystallised from aqueous alcohol: it foams and blackens at 214°. Found Na = 7·37 %. This was converted into the free sulphonylcystine as directed above and recrystallised: M.P. 215°. Found S = 20·64, N = 4·42 %.

When the filtrate from the sodium salt was made acid to litmus, naphthalene-sulphinic acid crystallised out in almost theoretical yield.

N-*Naphthalene-2-sulphonyl*-S-*benzylcysteine.*

The *S*-benzylcysteine used in this preparation was prepared according to the method of Suter [1895]. 2·12 g. were dissolved in 10 cc. *N* NaOH and shaken with 4·53 g. of naphthalene-2-sulphonic chloride dissolved in ether. 10 cc. of *N* NaOH were added at the end of each of 3 successive hours. At the end of 4 hours' shaking, the alkaline layer was separated and acidified with hydrochloric acid, the compound crystallising out: yield theoretical. It was recrystallised from 15 % aqueous acetic acid in long slender white silky needles: M.P. 132°. Found N = 3·7 %, S = 15·74 %, $C_{20}H_{19}O_4NS_2$ requires N = 3·49 %, S = 15·96 %.

Reaction between thiolacetic acid and naphthalene-2-sulphonic chloride.

2·76 g. of thiolacetic acid were dissolved in 10 cc. *N* NaOH and shaken with 2·76 g. of sulphonic chloride dissolved in ether. Further quantities of 5 cc. of NaOH were added in the usual way. At the end of 4 hours the alkaline layer was acidified thus precipitating naphthalene-2-sulphinic acid: yield = 0·92 g.; yield theoretically corresponding with the oxidation to dithioglycollic acid = 0·96 g.

The acid filtrate from the sulphinic acid was shaken twice with ether, the ethereal layer being dried over calcium chloride and the ether distilled off until about 10 cc. remained. The dithioglycollic acid crystallised from this in long needles; M.P. 107–8°. Found S = 34·8 %, requires 35·17 %.

Summary.

1. Both the reduced and oxidised forms of glutathione reduce naphthalene-2-sulphonic chloride to naphthalene-2-sulphinic acid in the presence of alkali. The yield of sulphinic acid in each case points to the formation of a disulphoxide of glutathione.

[1] The sodium was estimated according to the method of Coombs [1927]. My thanks are due to Mr Colwell who carried out the N, S and Na analyses mentioned in this paper.

2. Naphthalene-2-sulphonic chloride performs a twofold function towards cysteine: the oxidation to cystine and the consequent formation of dinaphthalene-2-sulphonylcystine, together with the appearance of the theoretical amount of naphthalene-2-sulphinic acid corresponding with this oxidation.

3. If the —SH group in cysteine is blocked as in *S*-benzylcysteine, then naphthalenesulphonic chloride gives a quantitative yield of *N*-naphthalene-2-sulphonyl-*S*-benzylcysteine, no sulphinic acid being formed.

4. Thiolacetic acid is oxidised to dithioglycollic acid by this reagent, naphthalenesulphinic acid appearing in theoretical yield.

I am indebted to Sir F. G. Hopkins for his kind interest and encouragement during the course of this work.

REFERENCES.

Abderhalden and Wybert (1916). *Ber. deutsch. chem. Ges.* **49**, 2468.
Coombs (1927). *Biochem. J.* **21**, 404.
Fischer (1915). *Ber. deutsch. chem. Ges.* **48**, 93.
—— and Bergell (1902). *Ber. deutsch. chem. Ges.* **35**, 3779.
Hopkins (1929). *J. Biol. Chem.* **84**, 269.
Miller and Smiles (1925). *J. Chem. Soc.* **127**, 224.
Otto (1891). *Ber. deutsch. chem. Ges.* **24**, 714.
Pirie (1930). *Biochem. J.* **24**, 51.
—— (1931). *Biochem. J.* **25**, 619.
Smiles and Gibson (1924). *J. Chem. Soc.* **125**, 176.
Suter (1895). *Z. physiol. Chem.* **20**, 562.
Thomas (1909). *J. Chem. Soc.* **95**, 342.

LIX. THE ENZYMIC HYDROLYSIS OF PHOSPHATIDES.

II. LYSOLECITHIN.

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(*Received March 7th, 1933.*)

Kyes [1903] showed that under the action of cobra venom lecithin was transformed into a substance having marked haemolytic properties. Delezanne and co-workers [1911; 1914] later demonstrated that the action was due to the presence of an enzyme in the venom, which was capable of removing the unsaturated fatty acid radical from lecithin. The substance so formed (the choline ester of monostearinphosphoric acid) possessed strong haemolytic properties. The appropriate name of lysolecithin was given to this material.

Levene, Rolf and Simms [1923] found it advantageous to allow the venom to act directly on emulsified egg-yolks, rather than on the lecithin isolated from eggs or other sources. In this way they prepared lysolecithin in a highly purified condition, free not only from non-lysing phosphatide material, but from the lysokephalin which ordinarily contaminates it.

It became desirable to prepare lysolecithin in this laboratory in connection with the investigation of the enzymic hydrolysis of lecithin [King, 1931] and other phosphatides. Difficulty was experienced, however, in obtaining the cobra venom. We were led to investigate the substitution of rattlesnake venom for that of the cobra through the suggestion of Mr R. H. Hutchinson of the Antivenin Institute of America, who kindly supplied us with the material free of charge.

Excellent yields of lysolecithin have been obtained by the use of this venom. The lysolecithin has been fractionated and prepared practically free from lysokephalin. Its haemolytic action has been tested against red blood cells, and its behaviour on hydrolysis by phosphate-liberating enzymes has been investigated.

Experimental.

Preparation.

The venom used was that of the fer de lance (*Bothrops atrox*), a variety of rattlesnake. The method of preparation followed was that suggested by Levene, Rolf and Simms, in which the venom is allowed to act directly on the egg-yolks. The yolks of 20 freshly laid eggs were freed as far as possible from the whites by gently washing the intact yolks in the hand under a slow current of warm water. After detaching the membranes, the yolks were emulsified with 600 cc. of 0·066 *M* Na_2HPO_4 buffer and the p_H adjusted to 7. 25 mg. of venom were dispersed in a little of the buffer and added to the emulsified egg material. A few drops of a mixture of chloroform and toluene were added as disinfectant to minimise bacterial growth. The venom was allowed to act on the phosphatides of the egg-yolks in a stoppered flask in a water-thermostat at 40° for 18 hours.

At the end of this time the enzyme action was stopped and the proteins were precipitated by pouring the mixture into 1000 cc. alcohol previously heated to 60°. The precipitated mixture was maintained at 50° during filtration on a large Büchner funnel into a suction flask. The residue on the funnel was removed and well washed with several portions of warm alcohol, using a total of 700 cc. Filtrate and washings were combined. After standing overnight a small further precipitate was filtered off and the clear yellow alcoholic liquid concentrated *in vacuo* to about 250 cc.

Great care is necessary during the concentration as the liquid froths badly. The operation is best carried out in a large distilling flask on a warm water-bath. The distillate can be caught in a second distilling flask, the side-arm of which is connected to the water-pump, at the end of the condenser. It is advisable to insert in the stopper of the large distilling flask a glass funnel carrying a stopcock. Through this the alcoholic solution can be fed, and by means of the stopcock the pressure can be so adjusted by the admission of air as to prevent frothing into the condenser.

The crude phosphatide material was precipitated by the addition of saturated aqueous cadmium chloride and a litre of acetone. A total of 25 cc. of cadmium chloride solution was added. Further addition gave no additional precipitate.

After standing in the ice-box overnight the cadmium salts of the mixed phosphatides had settled out. The clear supernatant liquid was poured off and the remainder centrifuged in 250 cc. cups. The precipitate was well washed by stirring up with acetone, shaking and re-centrifuging. At the end of five such operations the washings were water clear and the cadmium chloride salts almost pure white.

Acetone was sucked off by careful evacuation and the dry salts dissolved in the centrifuge-cups in about 150 cc. chloroform each. Ammonia gas was passed into the chloroform solution, well cooled in an ice pack for several hours until decomposition and precipitation of the cadmium ammonium chloride appeared to be complete. After centrifuging clear and washing the residue twice with chloroform, the combined centrifugates were filtered from a small amount of suspended material through a 15 cm. fluted paper. The filtrate was added in small portions to a 500 cc. distilling flask in which it was concentrated by careful warming and evacuation to a volume of about 50 cc.

The thick solution was poured and washed by successive small portions of chloroform into ether in two centrifuge-cups, and a heavy waxy white precipitate of mixed lysolecithin and lysokephalin separated.

Four preparations of this sort were made using different amounts of venom. The yields are indicated in Table I, together with the analytical figures for phosphorus (perchloric acid colorimetric method of King [1932], and nitrogen

Table I. *Yield of crude lysolecithin formed by the action of fer de lance venom on the lecithin of egg-yolk.*

	Amount of venom used mg.	Time of incubation hrs.	Yield g.	% P*	% N*
(1)	25	18	10·1	5·51	3·16
(2)	10	20	11·0	5·10	2·78
(3)	10	20	10·3	4·92	2·95
(4)	10	24	14·9	5·51	3·00

* Calculated for stearic lysolecithin P = 5·73 %; N = 2·59 %.
Calculated for palmitic lysolecithin P = 6·04 %; N = 2·72 %.
Calculated for stearic lysokephalin P = 6·44 %; N = 2·91 %.
Calculated for palmitic lysokephalin P = 6·84 %; N = 3·09 %.

(micro-Kjeldahl). The yields of crude lysolecithin with 10 mg. of fer de lance venom compared favourably with those obtained by Levene and Rolf using cobra venom.

Purification.

Lysolecithin prepared in this way is contaminated not only with lysokephalin, but also with small amounts of unattacked lecithin and kephalin. These are eliminated by repeated dissolution of the crude lysolecithin in chloroform followed by precipitation with ether, which retains the lecithin and kephalin in solution. Following this treatment the material was twice dissolved in the minimum amount of chloroform and precipitated by pouring into acetone. All operations were carried out in the centrifuge.

Iodine values (Hanus) determined on the original crude material averaged about 10, whereas the purified product absorbed almost no iodine. Amino-nitrogen estimations were attempted first by the Harris method [Hopkins, 1929]. This procedure consists in a titration of the amino-groups in an anhydrous medium (glacial acetic acid) with perchloric acid to a highly acid end-point, using brilliant cresyl blue as indicator. Whereas excellent results were obtained with simple amino-acids and with amino-ethyl alcohol (determined 23·05 % against a theoretical of 22·92 %), the method was found to be quite unsuitable when applied to phosphatides. The end-point was too broad to admit of any exact interpretation, and a precipitate appeared in the glacial acetic acid solution of the phosphatide when the perchloric acid was added. Accordingly, recourse was had to the Van Slyke method, the phosphatide being hydrolysed previous to the determination. 0·7 g. of the lysolecithin was boiled under reflux for 10 hours with 40 cc. of 4 % HCl. After cooling on ice the fats were filtered off and well washed. The combined filtrate and washings were neutralised to methyl orange, evaporated almost to dryness and then made up to 7 cc., and filtered through a fine small paper. 1 cc. of solution was taken as containing the nitrogen equivalent of 100 mg. of lysolecithin. 1 or 2 cc. were taken for amino-nitrogen determinations and 0·2 cc. for total nitrogen by micro-Kjeldahl. When analysed in this way the purified material showed an amino-nitrogen value corresponding to 23 % of the total nitrogen.

Fractionation.

An attempt was made to separate the lysolecithin from the lysokephalin by fractional precipitation from chloroform-ether solution following the method of Levene, Rolf and Simms [1924]. It was found, however, that the specific gravity of a chloroform-ether mixture containing moderate amounts of ether corresponded too closely with the material precipitated in it for it to be possible to effect any separation by centrifuging, and it was quite impossible to filter the mixture.

A fairly successful separation was accomplished by fractional precipitation from glacial acetic acid solution by ether and acetone. The lysokephalin appeared to be concentrated in the first fractions, some of those taken off later being almost pure lysolecithin.

25 g. of the mixed lysolecithin and lysokephalin were warmed with 50 cc. of glacial acetic acid for a few minutes at 60° until solution was complete. On cooling in the ice-box for 30 minutes a copious precipitate formed. This was centrifuged and 100 cc. of ether added to the precipitate while still wet with acetic acid. Almost complete solution resulted. To the centrifugate from this solution an excess of acetone was added to give a precipitate (fraction 1). On cooling in the ice-box for 2 days a further precipitate (fraction 1*b*) was formed.

To the glacial acetic acid centrifugate were added 3 volumes of ether, and a precipitate (fraction 2) removed after the mixture had stood in the ice-box. Fraction 2 was dried *in vacuo* over sodium hydroxide and well washed with ether. Fraction 3 was taken off after the centrifugate from fraction 2 had stood in the ice-box overnight. Acetone was added until cloudiness appeared and after chilling overnight fraction 4 was removed.

Addition of more acetone did not result in a further precipitate. The solution was accordingly concentrated by distillation *in vacuo* to about 25 cc. 25 cc. of ether and 50 cc. of acetone were added and a precipitate (fraction 5) removed after chilling. Fraction 6 was removed after the supernatant from 5 had stood 2 days in the ice-box. Acetone was added to 250 cc., the mixture well chilled and fraction 7 removed. The material remaining in solution was recovered by precipitation with cadmium chloride and subsequent decomposition with ammonia.

The several fractions were dried *in vacuo* over sodium hydroxide, washed with ether and dried over sulphuric acid. They were analysed by the methods mentioned above. Results of analyses are given in Table II. The figures for fraction 7 in particular correspond fairly closely with those required for pure lysolecithin. No attempt has been made to prepare the lysokephalin free from lysolecithin.

Table II. *Analyses of fractionated lysolecithin and lysokephalin.*

Fraction	P %	N %	Weight of fraction g.	$\frac{\text{Total N} - \text{amino-N}}{\text{Total N}} \times 100$*	Lysolecithin in fraction g.
1	5·26	2·75	2·4	58·2	1·39
1*b*	5·19	2·92	3·1	77·9	2·41
2	5·40	2·95	2·2	52·0	1·14
3	5·39	3·02	2·7	65·4	1·77
4	5·41	3·16	1·3	68·4	0·89
5	5·72	3·04	5·9	78·9	4·66
6	5·71	2·75	1·4	94·1	1·32
7	5·61	2·69	2·2	98·6	2·17
8	5·64	3·32	1·0	95·5	0·95
			22·2		16·70
Theoretical:					
Lysolecithin (stearic)	5·73	2·59		100	
Lysolecithin (palmitic)	6·04	2·72		100	
Lysokephalin (stearic)	6·44	2·91		0	
Lysokephalin (palmitic)	6·84	3·09		0	

* The figures in this column, representing the percentage of the total nitrogen which is non-amino-N, have been taken as equal to the percentages of lysolecithin in the mixtures.

The lysolecithin content of 22·2 g. of original material, calculated on the basis of the amino-nitrogen being 23 % of the total nitrogen, is 17·1 g. There is thus fair agreement between the calculated amount (17·1 g.) and that found (16·7 g.).

Haemolytic action of lysolecithin.

Haemolysis of red blood cells can be observed with the microscope by allowing a drop of an emulsion of lysolecithin in normal saline to run from the edge into a suspension of cells in saline under a cover slip. The cells appear to dissolve and disappear as the lysolecithin diffuses into the suspension.

The haemolysing activity was determined by observing the effect of different amounts of the lysolecithin on washed sheep cells. A 1 % solution was made by dissolving 25 mg. of the material (fraction 7) in 2·5 cc. of methyl alcohol. 1 cc. was diluted to 10 cc. to give a 0·1 % solution and 1 cc. of this diluted to 10 cc. to give 0·01 %. In this way a series of dilutions was made corresponding to different strengths of lysolecithin. For the tests 2 cc. of a 1 % suspension of cells in 0·9 % sodium chloride were added to 0·1 cc. of the lysolecithin solutions in small (0·8 × 7 cm.) test-tubes. The tubes were stoppered with cotton-wool and left in the ice-chest overnight.

Through the kindness of Dr P. A. Levene we were able to compare the activity of a sample of lysolecithin (containing 5 % amino-nitrogen) prepared at the Rockefeller Institute. The results of these tests are set out in Table III. Lysolecithin prepared in this laboratory (fraction 7) is referred to as (1), and that prepared at the Rockefeller Institute as (2). Complete haemolysis is abbreviated to CH, partial haemolysis, to Pa. H, slight haemolysis to Sl. H, and no haemolysis to No H. The haemolytic activities of the two samples appear to be almost identical. At the concentration (0·1 cc. of 0·01 %) which appears to be only partially effective in hydrolysing the 2 cc. of 1 % cells, the lysolecithin is at a final dilution of 1 in 200,000. If half this dilution be taken as the limit, it would appear that 1 part of lysolecithin will haemolyse about 1000 parts of red cells.

Table III. *Haemolysis of red blood cells by lysolecithin.*

(2 cc. of 1 % sheep cells added to 0·1 cc. of lysolecithin solution.)

Concentration of lysolecithin solution %	(1)	(2)
1·0	CH (0 hrs.)	CH (0 hrs.)
0·1	CH (0 hrs.)	CH (0 hrs.)
0·01	Pa. H (1 hr.)	Pa. H (1 hr.)
0·001	Sl. H (16 hrs.)	Sl. H (16 hrs.)
0·0001	No H (16 hrs.)	No H (16 hrs.)
Methyl alcohol	No H	No H

Enzymic hydrolysis of lysolecithin.

It has previously been shown [King, 1931] that there is an enzyme in kidney and intestinal mucosa which is capable of hydrolysing lecithin, liberating inorganic phosphate at a p_H optimum of 7·5. The enzyme appears to be contained in other tissues only to a slight extent. Bone, which is very rich in an enzyme hydrolysing primary esters of phosphoric acid, is relatively feeble in its action against lecithin. Lecithinase appears to act most rapidly at body temperature. It is fairly stable at a neutral reaction but is rapidly destroyed in an alkaline medium.

The behaviour of lysolecithin with both the intestinal and bone enzymes has been investigated. Fig. 1 shows the amounts of hydrolysis at different hydrogen ion concentrations and at different times when lysolecithin was subjected to the action of an aqueous extract of rabbit intestinal mucosa. An emulsion of the phosphatide was made by running 10 cc. of a 2 % alcoholic solution into 100 cc. hot water. To 10 cc. of this substrate in 25 cc. flasks were added 5 cc. veronal buffer [Michaelis, 1930] and 2·5 cc. of enzyme. Acid was added to give the desired p_H and water added to the mark. 3 drops of a mixture of chloroform and toluene were added to each flask, and they were placed in a water-thermostat at 37·5°. At stated intervals 5 cc. were removed from each

flask and added to 2 cc. of 10 % trichloroacetic acid. Inorganic phosphate (FP) was estimated in 5 cc. of filtrate. The increases in FP at the different times are plotted against p_H in Fig. 1. It will be seen that the maximum hydrolysis occurred at about p_H 7·8, which is a little higher than that reported for lecithin.

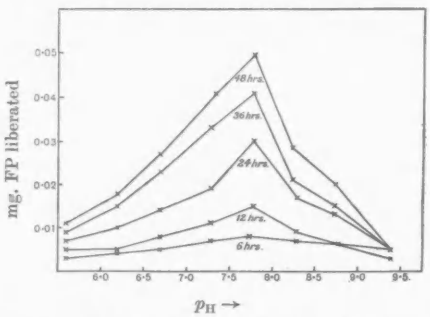

Fig. 1. Enzymic hydrolysis of lysolecithin (at 6, 12, 24, 36 and 48 hours).

The results of a comparative experiment on the rates of hydrolysis of lysolecithin and lecithin are given in Table IV. The substrate consisted of a 0·2 % emulsion of lysolecithin and a 0·25 % emulsion of lecithin. To 5 cc. of substrate

Table IV. *Hydrolysis of lysolecithin and lecithin by enzyme from small intestine.*

	Lysolecithin		Lecithin	
p_H	FP liberated	% hydrolysis	FP liberated	% hydrolysis
4·7	0·002	4·0	0·002	4·8
5·6	0·060	12·1	0·033	7·9
6·4	0·207	41·8	0·107	25·5
7·2	0·229	45·3	0·132	31·4
7·8	0·277	55·6	0·123	29·3
8·4	0·268	54·1	0·091	21·4
8·7	0·264	53·3	0·083	19·8
8·9	0·255	51·5	0·081	19·3

Table V. *Hydrolysis of lysolecithin and lecithin by bone enzyme.*

	Lysolecithin		Lecithin	
p_H	FP liberated	% hydrolysis	FP liberated	% hydrolysis
4·8	0·015	3·6	0·015	3·6
5·7	0·025	5·9	0·018	4·3
6·6	0·068	16·2	0·033	7·8
7·2	0·106	25·0	0·039	9·3
7·8	0·109	26·0	0·038	9·0
8·4	0·101	24·0	—	—
8·8	0·085	20·0	0·030	7·1
9·2	0·026	6·2	0·017	4·0

in test-tubes were added 6 cc. of buffer of the desired p_H and 2 cc. of enzyme from rabbit intestine. The action was stopped immediately in the controls by the addition of 2 cc. of 25 % trichloroacetic acid, and in the others after 48 hours at 37·5°. FP was estimated in 10 cc. of filtrate.

The FP liberated at the different p_H values was used, together with the organic phosphorus present at the beginning, to calculate the percentage hydrolysis which the phosphatide had suffered. It appears that lysolecithin is attacked to almost twice the extent that lecithin is, and at a slightly higher p_H optimum.

The bone enzyme was also investigated as to its effect on both lysolecithin and lecithin. Test solutions were put up as in the previous experiment, a 10 % extract of young rachitic rat bones being used as enzyme. Although lecithin was hydrolysed only slightly during 48 hours by this very active phosphatase preparation, lysolecithin was attacked to a considerable extent. The phosphatase activity of the bone extract measured against glycerophosphate was $2\frac{1}{2}$ times as great as that of the intestinal preparation, and yet the latter hydrolysed lysolecithin twice as much and lecithin $3\frac{1}{2}$ times as much as did the bone extract.

Summary.

1. Lysolecithin has been prepared by the action of the venom of the rattlesnake on the lecithin contained in egg-yolks.

2. The material so prepared has marked haemolytic properties.

3. Extracts of intestinal mucosa hydrolyse lysolecithin to a greater extent and at a faster rate than they do lecithin.

4. The bone enzyme, relatively inactive against lecithin, hydrolyses lysolecithin to a considerable extent.

5. There appears to be no relation between the phosphatase activity of a tissue extract and its ability to hydrolyse either lecithin or lysolecithin.

REFERENCES.

Delezanne and Ledebt (1911). *Compt. Rend. Acad. Sci.* **152**, 790.
—— and Fourneau (1914). *Bull. Soc. Chim.* (4), **15**, 421.
Hopkins (1929). *J. Biol. Chem.* **84**, 269.
King (1931). *Biochem. J.* **25**, 799.
—— (1932). *Biochem. J.* **26**, 292.
Kyes (1903). *Berl. klin. Woch.* **40**, 956, 982.
Levene, Rolf and Simms (1923). *J. Biol. Chem.* **55**, 743.
—— —— —— (1924). *J. Biol. Chem.* **58**, 859.
Michaelis (1930). *Biochem. Z.* **234**, 140.

LX. THE INFLUENCE OF OVARIAN AND ANTERIOR PITUITARY HORMONES ON CALCIUM METABOLISM.

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(*Received January 21st, 1933.*)

The belief that internal secretions associated with the reproductive organs have some influence on the calcium metabolism of the body is widely held. In particular the internal secretions of the ovary and of the anterior pituitary have been stated to have such an effect. Much of the evidence put forward is unsatisfactory.

Bell [1908], using methods of doubtful accuracy, reported a fall in the calcium content of human serum just before menstruation. Widdows [1922], on the other hand, could find no significant alteration in serum-calcium at any time during the menstrual cycle, while Sherman, Gillett and Pope [1918] and Bogert and McKittrick [1923] could find no evidence of change in the rate of calcium excretion during menstruation. More recently Frei and Emmerson [1930] have shown that there is no appreciable difference between the calcium contents of sera of cows at oestrus and dioestrus, although they hold the view that the chemical composition of serum is in some way connected with oestrus.

The results bearing on the influence of ovariectomy on the blood-calcium are entirely inconclusive. Blanchetière [1925] found an increased amount of calcium in the sera of women after ovariectomy and at the menopause. On the other hand Dalsace and Guillaumin [1925], also working with women, observed a fall in blood-calcium after ovariectomy. More recently Hogben and Charles [1932] have stated that ovariectomy in rabbits causes no change in serum-calcium.

Many workers have noted a fall in the serum-calcium of women during pregnancy [*cf.* Widdows, 1923; Mazocco and Moron, 1921; Plass, 1923]. This fall is probably due to the drain on the maternal tissues by the foetus which may demand as much as 0·6 g. calcium daily towards the end of gestation. It might be supposed of course that this mobilisation of maternal calcium is under control of a hormone, *e.g.* that of the corpus luteum. There is, however, no evidence that this is the case.

Luckhardt and Goldberg [1923] showed that parathyroidectomised dogs exhibited a recurrence of the symptoms of tetany at oestrus. This fact might suggest that the hormones concerned in the production of oestrus (one of the anterior pituitary hormones and oestrin) cause a lowering of the serum-calcium.

Mirvish and Bosman [1928] were among the first to try the effect of injection of extracts of the reproductive organs on the blood-calcium in experimental animals and in man. They claimed that the injection of oily solutions of extracts of liquor folliculi, ovary, corpus luteum and placenta, made by methods based

on those introduced by Herrmann and Fränkel [1915] for oestrus-producing extracts, caused a lowering of the blood-calcium of rabbits. Since the effect was the same whether males, normal, pregnant or ovariectomised females were used, they were inclined to the view that the oestrus-producing hormone was not the agent responsible, but rather, a different hormone present in the extract. Subsequently they stated that similar effects could be observed on injecting the extracts into human males and females. Reiss and Marx [1928] have reported similar lowerings of the blood-calcium of rabbits as a result of injection of a commercial ovarian extract ("homovar"). Later work by Frei and Emmerson [1930], in which they injected a commercial oestrin preparation, "progynon," into cows, showed that there was a slight fall in serum-calcium (0·5 mg./100 cc.). Mirvish and Bosman, in later work [1929], showed that extracts of testis and adrenal cortex also possessed this calcium-lowering potency.

A fresh aspect of the problem has been opened up by the recent work of Hogben and Charles [1932]. These workers have shown that the injection of saline extracts of the anterior lobes of ox pituitaries produced a marked fall in serum-calcium when injected into normal female rabbits. That the effect was a direct one of the anterior lobe, and not an effect due to the stimulation of the ovary by the extract, was clear from the fact that similar falls were observed in ovariectomised rabbits. It was also shown that a few hours after coitus there was a fall in the serum-calcium of female rabbits which was followed by a rise coincident with the time of ovulation and a second fall which subsided by the time the corpus luteum was fully formed. These latter effects appear possibly to be due to the action of the anterior pituitary also. Frei and Emmerson [1930] have shown that there is a slight rise in serum-calcium 8 hours after the injection of anterior pituitary extract into cows.

Cannavo [1932], on the other hand, has shown that injections of active anterior pituitary extracts into man, the dog and the rabbit cause little change in the calcium content of the serum.

The present work, following on that of Mirvish and Bosman and of Reiss and Marx was an attempt to find out if the alleged lowerings of serum-calcium found by these workers on injection of ovarian, corpus luteum and follicular extracts into rabbits were due to oestrin or other substances present in the ovary. If they were due to oestrin it was thought that this might provide a useful method for the assay of the latter.

Experimental.

The first step, then, before injection of crystalline oestrin was the repetition of the above work with ovarian extracts. Extracts of whole ovary, residual ovary and corpus luteum were accordingly made by repeatedly boiling the minced tissues with alcohol, evaporating and extracting the residue with methyl alcohol. The methyl alcohol fraction on evaporating to dryness and dissolving in olive oil was used for injection without further purification.

Serum-calcium determinations were made by the Trevan-Bainbridge [1926] method and inorganic phosphorus by the Fiske-Subbarow [1925] method. All determinations were done in duplicate.

The animals first used for the tests were normal buck and ovariectomised doe rabbits previously kept on a diet of cabbage and oats for several weeks. The animals were bled on alternate days for a week or so previous to injections to find out if a reasonable constancy of serum-calcium from day to day could be expected. The results of these determinations before and after injection of

the extracts are shown in Fig. 1, and are unsatisfactory for they do not show any great difference in serum-calcium after injection nor is the serum-calcium very constant from day to day but appears to depend to a large extent on diet, cabbage alone causing it to increase.

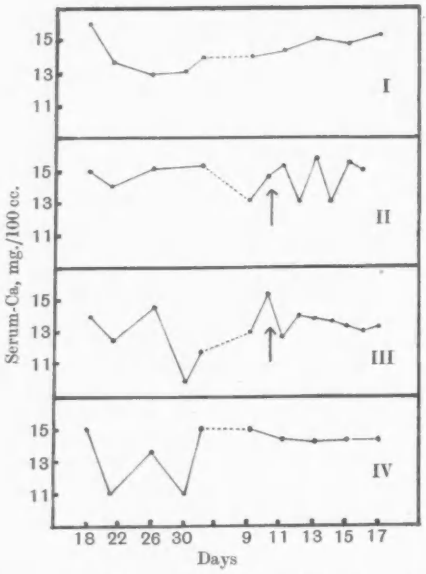

Fig. 1. Ovarian extract injected into rabbits II and III = 300 g. fresh substance; blood samples taken 12 and 24 hours after injection. I and IV controls. Rabbits I and II bucks; III and IV does.

Since these experiments, two papers dealing with the influence of diet on serum-calcium have appeared [Dupré and Semeonoff, 1931; Bourne and Campbell, 1932]. These authors show conclusively that feeding cabbage causes the serum-calcium level to rise and that of phosphorus to fall; whereas feeding oats causes the calcium content to decrease and that of phosphorus to rise. Since it is impossible to make a rabbit eat the same proportions of cabbage and oats per day, the fluctuations in serum-calcium observed by us and ascribed by other authors to the effects of injections, bleeding *etc.* are readily explained. That rabbit serum-calcium can vary from 10 to 18 mg./100 cc. under the influence of diet shows that unless rigid precautions are taken with regard to feeding the interpretation of changes in serum-calcium may be misleading. It has been suggested by Dupré and Semeonoff that calcium determinations on rabbit serum should be done after an 18 hours' fast, when some kind of equilibrium between the calcium and phosphorus has been attained.

Some authors report changes in rabbit serum-calcium when blood is taken on successive days. Charles [1931] reports a rise, Clark [1921] and Stewart and Percival [1928] report a fall, whereas Mirvish and Bosman [1928] state that repeated bleeding has no effect on serum-calcium in rabbits. As shown above no deductions about this can be drawn unless diet is rigidly controlled. Hogben and

Charles [1932] suggest the use of numbers of parallel control and experimental groups of animals, the blood of control and experimental groups being taken at the same time to obviate the complication due to successive bleedings. There is no doubt that this is an improvement over the use of single experimental animals. But, as is shown in Table I, the serum-calcium of 10 normal rabbits previously

Table I. *Rabbits. Serum-calcium of 10 normal doe rabbits kept under standard conditions for 3 weeks. Cabbage and oats in morning, 12 noon, bleeding from 3 to 5 p.m.*

31. i. 32	Rabbit No.	Calcium mg./100 cc.	Rabbit No.	Calcium mg./100 cc.
	1	14·22	7	14·77
	2	15·99	8	15·65
	4	16·65	9	15·00
	5	17·05	10	13·93
	6	16·15	15	16·22

kept on a standard diet for 3 weeks, all bled at the same time, varies from 13 to 17 mg./100 cc. Thus unless very large numbers of animals in each group are used, no values of serum-calcium, unless outside the limits of normal variation, can be taken to prove that any particular condition is responsible for a difference in the serum-calcium level.

Some other animal having a more constant serum-calcium was sought for, and preliminary experiments on dogs showed that they were far less subject to daily and dietary variations. Extracts of residual ovary and corpus luteum were accordingly made and injected subcutaneously into dogs, blood samples being taken from leg veins at various intervals after injection. As the results in Figs. 2–4 show there is no appreciable change in serum-calcium after injection.

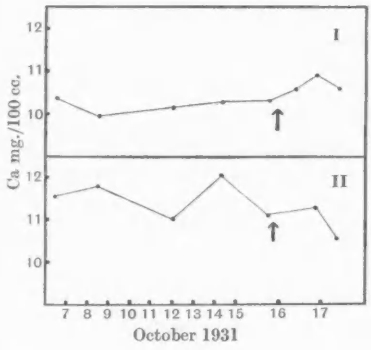

Fig. 2.

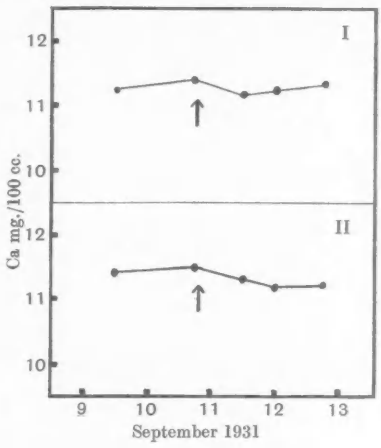

Fig. 3.

Fig. 2. Ovarian extract and dog serum-Ca. I injected; II control. Injection = 400 g. fresh substance. Normal bitches.

Fig. 3. Injections of ovarian extract into 2 ovariectomised bitches. I injected extract equal to 300 g. fresh substance; II injected olive oil only.

It was then thought that if an animal having a regular oestrous cycle was used it might be possible to demonstrate changes in calcium metabolism during the cycle due to intermittent secretion of oestrin by the ovary. Thus it was decided to carry out experiments on rats, since these animals have a regular

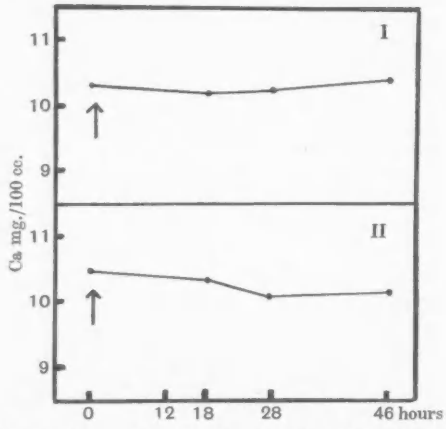

Fig. 4. Effect of injection of corpus luteum extract equal to 300 g. fresh substance into ovariectomised bitch I. II was an ovariectomised control injected with olive oil only.

oestrous cycle every 5 days which can be followed by means of the vaginal smear technique. Batches of rats kept on standard diet were killed off at various stages of their oestrous cycle and their blood drawn by cardiac puncture. Calcium determinations at oestrus when uterine distension is at a maximum and at dioestrus show no appreciable difference.

Table II. *Serum-calcium of normal rats.*

Oestrus					Dioestrus				
Date	No.	Serum-Ca	How killed	Diet	Date	No.	Serum-Ca	How killed	Diet
30. xi. 31	16	13·9	$CHCl_3$	Stock	30. xi. 31	19	13·6	$CHCl_3$	Stock
30. xi. 31	11	13·3	,,	,,	30. xi. 31	18	13·85	,,	,,
6. xi. 31	38	11·7	Ether	,,	6. xi. 31	32	11·45	Ether	,,
13. i. 32	200	12·2*	,,	,,	8. xi. 31	36	13·95	,,	,,
18. i. 32	267	17·0†	,,	,,	8. iv. 32	397	10·75	Chloralose	,,
18. i. 32	268	17·8‡	,,	,,	8. iv. 32	402	10·9	,,	,,
4. ii. 32	242	13·2	,,	,,	13. iv. 32	454	11·15	,,	α
7. iv. 32	257	10·7	Chloralose	,,	27. iv. 32	478	10·6	Amytal	α
8. iv. 32	397	—	—	—					
3. v. 32	476	11·0	Amytal	α					
2. iv. 32	474	11·0	,,	—					

The stock diet contained 1·2 % calcium 0·3 % phosphorus.
The stock diet α contained 1·2 % calcium 0·8 % phosphorus.
* Injected with 6 units parathormone 9 hours.
† Injected with 20 units parathormone 6 hours.
‡ Injected with 20 units parathormone 12 hours.

A group of rats was then injected with extract of bovine anterior pituitary, injections being made daily for 5 days. The animals were killed 7 days after the first injection when the corpora lutea were fully grown as was found on dissection

of the ovaries. The blood-Ca exhibited no appreciable difference from that of controls killed at the same time. The condition of the ovaries of these animals was similar to that in pseudopregnancy which occurs after copulation with a sterile buck.

Table III. *Injection of anterior pituitary extract into rats.*

		Control	
No.	Ca mg./100 cc.	No.	Ca mg.
401	11·9	435	11·85 (oestrus)
405	—		
406	10·65		
431	12·1		

Rats Nos. 401, 405, 406 and 451 on dissection showed ovaries to be full of corpora lutea.
Injections were carried out on 5 consecutive days, rats being killed on 7th day. The rats were smeared every day and it was noted that in each case the oestrous cycle was inhibited after the 2nd day.

It was then decided to study the effect of pregnancy and pseudopregnancy on the serum-calcium and phosphate levels in rabbits using the technique of 18 hours' starvation before taking blood samples. Table IV shows that a group of 10 doe rabbits, some in oestrus and some in resting condition exhibit a very small range of variation in serum-calcium. Of these rabbits previously kept caged on a standard diet for 3 weeks, 4 copulated twice with a vasectomised buck and 2 with a normal buck, the remainder being used as controls. Blood samples were taken from these animals 8 days after copulation, when the corpus luteum was assumed to have reached a reasonable degree of activity and growth, together with blood samples from controls. As Table V shows, the serum-calcium values are not materially different from each other or from those in Table IV which were values for normal females.

Table IV. *Serum-calcium of 10 normal doe rabbits kept under standard conditions for 4 weeks and starved 18 hours before bleeding.*

11. iii. 32	Rabbit No.	Ca	Rabbit No.	Ca
	1	14·1	7	13·5
	3	13·6	8	14·5
	4	14·0	9	14·0
	5	13·6	11	14·1
	6	13·5	12	14·2

This shows the effect of 18 hours' starvation on the reduction of variation of the serum-calcium.

Table V. *Serum-calcium and phosphorus of 4 pseudopregnant, 2 pregnant and 3 normal immature doe rabbits.*

3. iii. 32	Rabbit No.	Ca	P
	8 pseudopregnant	13·7	4·2
	P_1 ,,	13·4	4·3
	P_2 ,,	13·5	4·0
	P_3 ,,	13·35	3·9
	P_4 9 days pregnant	13·9	4·3
	P_5 ,,	12·3	4·4
	6 immature control	12·8	4·5
	12 ,,	13·5	4·1
	8 ,,	13·7	4·0

The observation by Luckhardt and Goldberg [1923] that oestrus brings about recurrence of tetany in parathyroidectomised dogs suggested that the parathyroids might mask or compensate for changes in serum-calcium due to secretion of oestrin. Experiments were therefore carried out on rats which had been parathyroidectomised. A group of these animals all receiving the same diet were killed at stages of oestrus and dioestrus and their blood drawn and analysed for calcium and phosphate. The serum-calcium values for the oestrus rats ranged from 5·4 to 8·4 mg./100 cm. with an average of 6·71 and that of the dioestrus ones from 6·0 to 8·4 mg./100 cc. with an average of 8·0 mg. This difference in view of the range of variation does not appear to be significant.

Table VI.

Oestrus					Dioestrus				
No.	Date of killing	Ca	P	Days after operation	No.	Date of killing	Ca	P	Days after operation
442 C	12. iv. 32	5·4	14·4	21	444 C	12. iv. 32	6·0	10·8	21
445 E	12. iv. 32	7·45	10·9	21	441 E	12. iv. 32	8·40	7·8	21
450 C	18. iv. 32	8·1	11·6	25	453 C	18. iv. 32	8·3	8·3	25
440 A	18. iv. 32	7·28	16·0	25					
468 C	19. iv. 32	8·5	11·6	25	467 C	19. iv. 32	8·8	8·5	25
443 A	25. iv. 32	7·27	11·9	34	446 A	25. iv. 32	8·43	10·1	34
449 A	25. iv. 32	6·50	14·2	34	456 A	25. iv. 32	7·50	12·9	34
479 A	27. iv. 32	6·7	—	27	478 A	—	—	—	27
473 A	3. v. 32	7·5	—	32	462 A	2. v. 32	8·4	—	37
					460 A	2. v. 32	7·8	—	37
471 A	3. v. 32	6·75	—	38	465 A	3. v. 32	7·6	—	38
470 A	6. v. 32	8·4	13·5	41	466 A	6. v. 32	8·85	11·5	41
	Mean	6·71	12·9				8·0	9·9	

Serum-calcium of parathyroidectomised rats:
A = killed amytal anaesthesia intraperitoneally 0·02 g.
C = killed chloralose anaesthesia intraperitoneally 0·05 g.
E = killed ether.

Another group of rats was ovariectomised at about the beginning of maturity and a week later parathyroidectomised. None of these animals died from tetany during the following week. Half of them were then injected with approximately 6000 m.u. of cryst. trihydroxyoestrin in 4 doses at 12-hour intervals and all

Table VII. *Effect of trihydroxyoestrin injections on ovariectomised parathyroidectomised rats.*

20. v. 32	No.	Ca mg./100 cc.	Condition of uterus
	714	5·3	Well distended
	715	6·15	,,
	719	6·8	Moderately distended
	721	6·4	Well distended
	722	6·0	Moderately distended
Controls:	724	6·2	Undistended
	725	6·25	,,
	726	4·60	,,
	727	5·8	,,

Animals were ovariectomised 5. v. 32 and parathyroidectomised 11. v. 32.
Oestrin was given in 4 equal doses of 1500 m.u. totalling 6000 m.u.
They were killed by intraperitoneal injection of 0·01 g. amytal. Their diet up to 6 hours before death when they were starved contained 1·2 % Ca, 0·86 P.
Blood taken by cardiac puncture about 10–15 minutes after injecting anaesthetic.

were killed 30 hours after the last injection, at which time the vaginal smears of those injected with oestrin were cornified and the uteri on dissection well distended. The serum-calcium of the oestrin-injected varied from 5·3 to 6·8 mg., average 6·13 mg./100 cc. and of the ovariectomised parathyroidectomised controls from 4·6 to 6·2 mg./100 cc., average 5·7 mg. This difference is also not significant. The wide range of variation of serum-calcium in parathyroidectomised rats may be accounted for possibly by incomplete removal of parathyroids.

Discussion.

As already pointed out by Bourne and Campbell and Dupré and Semeonoff the serum-calcium of rabbits is very variable under normal conditions of diet and estimations must be interpreted with caution, unless the food intake is controlled. It is not sufficient to give the same amount of green stuff and oats or bran daily or either of these foodstuffs singly, as variations in serum-calcium may still exist. The only satisfactory way is to withhold food for 18 hours before the withdrawal of blood.

In dogs variations in serum-Ca due to variation in diet are small. In rats, as Howland and Kramer [1932] have shown, the percentage of calcium and phosphate in the food influences the amounts of calcium and phosphorus in the serum, but the latter are moderately constant for the same diet.

From the above experimental results it can only be concluded that oestrin and corpus luteum hormone cause no gross change in serum-calcium in rabbits, dogs and rats.

Summary.

1. Ovarian and corpus luteum extracts injected into rabbits and dogs produce no significant change in their serum-calcium levels.

2. In rabbits pregnancy and pseudopregnancy produce no demonstrable change in serum-calcium after 8 days.

3. Serum-calcium levels of rats in oestrus and dioestrus are not appreciably different.

4. Injections of anterior pituitary extract into rats sufficient to cause luteinisation of ovaries caused no change in their serum-calcium.

5. The serum-calcium values in oestrus and dioestrus of parathyroidectomised rats are very variable and no significant difference can be observed.

6. Injections of crystalline trihydroxyoestrin sufficient to produce uterine distension and vaginal cornification in ovariectomised parathyroidectomised rats do not produce any marked difference in serum-calcium from controls.

Thanks are due to Prof. J. C. Drummond and Dr G. F. Marrian for their interest and advice and to Dr Hoskins for her assistance in parathyroidectomising the rats. The expenses of the research were defrayed from a grant to Dr Marrian by the Medical Research Council, to whom the author wishes to express his gratitude.

REFERENCES.

Bell (1908). *Proc. Roy. Soc. Med.* **2**, 291.
Blanchetière (1925). *Compt. Rend. Soc. Biol.* **92**, 491.
Bogert and McKittrick (1923). *J. Biol. Chem.* **54**, 365.
Bourne and Campbell (1932). *Biochem. J.* **26**, 183.
Cannavo (1932). *Biochem. Z.* **245**, 234.
Charles (1931). *Quart. J. Exp. Physiol.* **21**, 81.
Clark (1921). *J. Biol. Chem.* **49**, 482.
Dalsace and Guillaumin (1925). *Compt. Rend. Soc. Biol.* **93**, 1209.
Dupré and Semeonoff (1931). *J. Biol. Chem.* **94**, 341.
Fiske and Subbarow (1925). *J. Biol. Chem.* **66**, 375.
Frei and Emmerson (1930). *Biochem. Z.* **226**, 355.
Herrmann and Fränkel (1915). *Monatsh. Geburtsh. Gynakol.* **41**, 1.
Hogben and Charles (1932). *J. Exp. Biol. Med.* **9**, 139.
Howland and Kramer (1932). *J. Nutrition*, **5**, 39.
Luckhardt and Goldberg (1923). *J. Amer. Med. Assoc.* (1), 79.
Mazocco and Moron (1921). *Compt. Rend. Soc. Biol.* **85**, 692.
Mirvish and Bosman (1928). *Quart. J. Exp. Physiol.* **18**, 1.
—— —— (1929). *Brit. J. Exp. Biol.* **6**, 350.
Plass (1923). *J. Biol. Chem.* **56**, 291.
Reiss and Marx (1928). *Endokrinologie*, **1**, 181.
Sherman, Gillett and Pope (1918). *J. Biol. Chem.* **34**, 372.
Stewart and Percival (1928). *Physiol. Rev.* **8**, 283.
Trevan and Bainbridge (1926). *Biochem. J.* **20**, 423.
Widdows (1922). *Lancet*, ii, 1037.
—— (1923). *Biochem. J.* **17**, 34.

LXI. AN ABSORPTION APPARATUS FOR THE MICRO-DETERMINATION OF CERTAIN VOLATILE SUBSTANCES.

I. THE MICRO-DETERMINATION OF AMMONIA.

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(*Received January 9th, 1933.*)

1. The apparatus and method.

While several methods already exist for the estimation of ammonia, that arising out of the present study has the advantage (besides applying in principle to the analysis of other substances) of combining extreme simplicity with high accuracy in the estimation of very minute quantities. It also permits the convenient carrying out at the one time of a large number of analyses. With the apparatus the error of a single estimation expressed as the coefficient of variation need not exceed 0·5 %, and this degree of accuracy is practically maintained with estimations of ammonia as low as 0·01 mg. The mean value itself does not fall below 99·5 % of the theoretical, and the delicacy of the method is such that the analysis of the blood-ammonia comes within its range.

The principle of the method consists in the absorption by simple gaseous diffusion of the volatile substance from one chamber where it exerts a certain tension into another where its tension is zero on the surface of an absorbing fluid. On a first consideration this would seem a very slow process and scarcely lending itself to practical estimations. It has indeed been used by some previous workers—*vide* Neuberg [1911] and Durig [1907]—who with a very different technique from that here described were obliged to wait upwards of 48 hours for the conclusion of the analysis and even then obtained somewhat low results.

The principle of the method may be extended to the estimation of any volatile substance the tension of which is reduced to zero on the surface of an absorbing fluid. It has been successfully extended to the estimation of chloride (as chlorine)—being very suitable for the estimation of very minute quantities—and to carbon dioxide.

The apparatus. The apparatus is of a very simple kind and consists of a glass container resembling a small Petri dish with thick walls, within which is a second chamber formed by a circular wall of glass arising from the floor of the outer chamber. The wall of the inner chamber is about half the height of the outer. The apparatus is constructed of thick glass, which (for the present investigation) should give off no alkali to the acid in the middle chamber. The upper edge of the outer wall has a ground surface and is covered during the absorption with a square glass lid on which is smeared a small amount of fixative such as vaselin or a mixture of vaselin and paraffin wax, and in this

way quite readily makes an air-tight compartment. The dimensions of a unit at present available from the manufacturers are given in the diagram (Fig. 1).

The method. In the investigation of the ammonia absorption 1 cc. of standard acid is introduced into the inner chamber of a clean dry unit from a 2 cc. standard burette from which it may be very accurately delivered (with a coefficient of variation which need not exceed 0·1 %). Where extremely weak acids are used—less than 0·001 *N*—a pipette simply constructed from a pyrex tube is advisable. Into the outer chamber is delivered a measured quantity of the fluid to be analysed (not exceeding 3 cc.) by means of an Ostwald pipette. The lid is suitably smeared with the fixative and placed in position. The apparatus is now slightly tilted—most conveniently by resting one edge on a second glass lid. The tilting collects the fluid in the outer chamber away from where the alkali is introduced. The lid is horizontally displaced so that a small opening appears into the outer chamber sufficient to allow the introduction of the tip of a pipette containing a measured quantity of alkali—usually 1 cc. of saturated potassium carbonate. The alkali is run in quickly and the lid replaced. The unit apparatus (which will be subsequently alluded to simply as a "unit") is now gently rotated about 10 times to mix the fluids in the outer chamber and then placed in an incubator at a temperature not exceeding 40°, or if the fastest absorption time is not required is left aside at room temperature. After a suitable time for the absorption the lid is detached, and titration of the acid gives a measure of the ammonia absorbed.

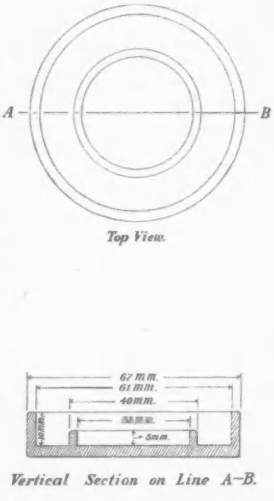

Fig. 1. Plan of apparatus as described in text. (Apparatus of this standard size is available from T. H. Mason, 5 Dame Street, Dublin.)

A few initial experiments carried out by one of us in the above manner revealed a surprising rapidity in the absorption of ammonia so that determinations could be carried out with great ease in a comparatively short time. Where a large number of determinations are required—and sets of 25 to 50 can be conveniently done simultaneously—the average time per estimation may be reduced to a few minutes by suitable arrangement of the periods of filling and titration. The absorption itself requires no attention and may be conveniently carried out at room temperature—the time for the absorption with 1 cc. volumes being then about 1½ hours.

The absorption time depends upon the volume of fluid in the outer chamber, the quantity and kind of alkali used to liberate the ammonia and the temperature at which the absorption is carried out.

2. Various factors influencing the absorption rate.

These were investigated in detail and have mostly an application to other methods of absorbing the ammonia.

(*a*) *The time-absorption curve at constant temperature.*

In the investigation of the time course of the absorption the following procedure was adopted. Into the inner chamber was run 1 cc. of 1·2 *N* H_2SO_4 and

into the outer 1 cc. of 0·1 N ammonium chloride solution as already described, and the unit incubated at 38° for 15 minutes to ensure its reaching this temperature. It was then removed and 1 cc. of N NaOH—at a temperature of 38°—was quickly introduced into the outer chamber in the manner already described. After mixing the unit was again returned to the incubator. A large number of experiments was carried out, and the acid in the central chamber was titrated with 0·05 N alkali at different time intervals. Suitable blanks were also included. The Ostwald pipette used was subsequently standardised against a 2 cc. standard burette. The results obtained are illustrated in Fig. 2, in which the curve is drawn from the equation

$$\frac{1}{t}\log\frac{a}{a-x} = A_1 = 0{\cdot}0189 \qquad \text{.....(1),}$$

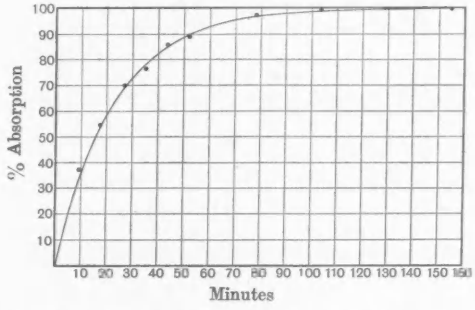

Fig. 2. The curve gives the percentage absorption of ammonia in 1 cc. of 0·12 N sulphuric acid in the central chamber. In the outer chamber was placed 1 cc. of 0·1 N ammonium chloride *plus* 1 cc. of N NaOH. Temperature, 38°.

where x is the quantity absorbed in t minutes and a the total quantity of ammonia introduced into the outer chamber at zero time. In the figure a is represented as 100. A_1 is a constant for the particular absorption carried out at constant temperature. The curve clearly fits the observed points and has the familiar form of that for a unimolecular reaction. It may be derived from the relation

$$\frac{dx}{dt} = 2{\cdot}30\ A_1\ (a - x) \qquad \text{.....(2),}$$

where dx/dt is the rate of change of the quantity of ammonia in the outer chamber and is proportional to the amount contained therein at any time t. It will be seen that A_1 is the absorption rate per minute per unit quantity of ammonia in the outer chamber—divided by 2·30. It is to be noted that this quantity is dissolved in 2 cc. of fluid. The constancy of A_1 under similar conditions of volume, temperature, *etc.*, makes it a very convenient figure for investigating the effect on the absorption rate of changing these various factors.

(*b*) *The effect of temperature on the absorption rate.*

In this investigation estimations of the absorption rate or A_1 value (equation 1) were carried out as in the previous method for examining the time-absorption curve. The temperature of the units and fluids was suitably varied by altering the temperature of the incubator, *etc.*, 30 minute periods were allowed as a rule for the absorption. The temperature effect found may be stated as an increase of 3·9 % per degree in the absorption rate, which is nearly twice as fast at 38° as at ordinary room temperature. Altering the concentration of the ammonia

in the outer chamber has no influence on the temperature effect. When however the fluid in the outer chamber is 50 % saturated with potassium carbonate the increase in the absorption rate per degree is reduced to 2·9 %.

(*c*) *The effect of altering the* p_H *of the fluid in the outer chamber.*

If we consider the rate of absorption (A_1) at high p_H values where all the ammonia may be considered present as gas and the rate (A_2) at a p_H where a proportion only exists as gas, the ratio of these two rates will be the ratio of the tensions of ammonia gas developed by unit quantity of total ammonia in each case. This however is clearly the ratio of the degrees of dissociation of the ammonium ions if we regard these as dissociating into ammonia gas and hydrogen ion [Michaelis, 1929]. For the absorption rate A_1 this dissociation is 1·00 and for A_2 it is α so that

$$\frac{A_2}{A_1} = \alpha \qquad \text{.....(3).}$$

The experimental values of α should correspond with the theoretical and the correspondence is practically exact when we take the p_K value for the dissociation of the ammonium ion as 9·44 (the experimental values of α lying along the theoretical curve of α against p_H as obtained from equation 4). The effect of changing the p_H value of the fluid in the outer chamber may be therefore summarised in the statement that the absorption rate is proportional to the degree of dissociation of the ammonium ion, as determined by the following equation

$$p_H = 9{\cdot}44 + \log \frac{\alpha}{1-\alpha} \qquad \text{.....(4).}$$

The experimental determination of the absorption rate at different p_H values was carried out in the following manner. Into the inner chamber was introduced 1 cc. of 0·001 *N* sulphuric acid and into the outer 1 cc. of 0·001 *N* ammonium sulphate. After the adjustment of the lid, *etc.*, 2 cc. of a Sørensen borate solution were introduced into the outer chamber. The fluids were mixed by rotating 5 times and then left aside at room temperature. After a suitable time (30–100 minutes) the contents of the inner chamber were titrated with 0·0005 *N* CO_2-free alkali, using a small quantity of the special indicator (methylene blue and methyl red mixture described at the end of the paper). Suitable blanks were carried out.

The p_H of *N* ammonia is 11·6 (approx.) at which 99·5 % of the total ammonia may be considered present as gas. The p_H of *N*/10 ammonia is approximately 11·1 at which 98·5 % of the total ammonia may be also considered present as gas. *N* ammonia may therefore be used in determining the effect of temperature, saline concentration, *etc.*, on the absorption rate.

(*d*) *The effect of salt addition on the ammonia absorption.*

The effects of various salts on the ammonia tension in pure ammonia solutions have been exhaustively investigated by Gaus [1900] and by Abegg and Riesenfeld [1902]. An investigation was carried out with the present apparatus to find the effect on the absorption rate of introducing saturated solutions of those salts found to be most potent in their effect in increasing the ammonia tension. 1 cc. of pure *N* ammonia was introduced into the outer chamber containing 1 cc. of a saturated solution of the salt investigated—the inner chamber containing 1 cc. of *N* sulphuric acid. A period of 10 or 15 minutes was allowed for the absorption—the whole procedure being conducted at 38°. The results obtained are listed in Table I, which shows that as a result of salt addition the absorption rate of the ammonia can be increased to over three times by using either potassium metaborate or potassium carbonate. Potassium fluoride and 40 % KOH also cause a very marked increase. Alkaline potassium phosphate causes an increase of 106 %.

Table I.

Substance investigated	Concentration of substance in mixed fluid at 38°	Absorption rate at 38° (A_1 value, see equation (1) in text)	Calculated % increase in ammonia tension in outer chamber	% increase in NH_3 tension found by Abegg and Riesenfeld using 0·5 N sols. of the substances in col. 1
KBO_2	½ sat.	0·054	218	24
K_2CO_3	,,	0·050	194	27
KF	,,	0·045	165	19
K_2HPO_4	,,	0·035	106	17
$K_2C_2O_4$	,,	0·024	41	15
KOH	20 %	0·041	141	20
NaOH	,,	0·037	118	13
NaOH	0·5 N	0·019	12	13
Water		0·017	0	0
Glucose	25 %	0·014	− 17	

(*e*) *The effect of the fluid volume in the outer chamber.*

The absorption rate of the ammonia is clearly dependent on the ammonia tension of the gas in the outer chamber, which in turn from Henry's law is proportional to the amount of gas dissolved per unit volume, and Henry's law holds for weak ammonia solutions [Mellor, 1928]. The absolute absorption rate is thus proportional to the concentration of ammonia in the outer chamber. The relative or percentage absorption rate—which may be expressed as the absolute absorption rate divided by the quantity of ammonia in the outer chamber—is consequently inversely proportional to the volume of fluid contained therein. The relative absorption rate from equation (2) is

$$2{\cdot}30A_1 \text{ (where } A_1 = \frac{1}{t}\log\frac{a}{a-x}\text{, from equation 1)},$$

and from Table II is clearly inversely proportional to the volume of fluid from which the ammonia is being absorbed.

Table II.

Quantity of ammonia in outer chamber (millimols)	V = volume of fluid in outer chamber cc.	Time allowed for absorption mins.	A_1 value = $\left(\frac{1}{t}\log\frac{a}{a-x}\right)$	A_2 value = $2{\cdot}30\,A_1\,V$
1·0	1·0	16	0·0153	0·0352
1·0	2·0	26	0·0080	0·0368
1·0	3·0	24	0·0056	0·0386
1·0	4·0	22	0·0041	0·0377

Temperature = 18·5°.

Two estimations were carried out for each volume. The A_2 value represents the calculated absorption rate in millimols per minute per unit concentration of the ammonia from the outer chamber, unit concentration being here 1 millimol per cc.

The practical result—apart from the effect of salt addition—is that increasing the volume of fluid in the outer chamber increases the time for 99·5 % absorption in direct proportion. The addition of saturated alkaline solutions such as saturated potassium carbonate will therefore produce two opposite effects, one due to the pure salt action in increasing the tension of the ammonia and the other due to the increased volume. There should thus be an optimum volume of addition of the saturated solution. For potassium carbonate this occurs at an addition of 0·7 cc. of the saturated fluid to 1 cc. of the fluid to be analysed. However both theoretically and practically there is in fact a fairly wide range of

addition over which the absorption rate is almost unchanged. Thus with 0·5, 1·0 and 1·5 cc. additions of saturated carbonate to 1 cc. the absorption rates are 0·031, 0·032, 0·030 respectively, expressed as A values (equation 1).

(*f*) *The effect of the fluid volume in the inner chamber.*

Since on the surface of the acid in the central chamber the ammonia tension is zero, we should expect no change on altering its volume except the slight one of diminishing the distance of passage of the molecules. A series of experiments was carried out with 1 cc. of acid in the inner chamber and a similar set under the same conditions using 2 cc. of acid, there being present in each set 2 cc. of pure ammonia solution in the outer chamber. In the first group the absorption rate (A value) was 0·0094 and in the second 0·0099, the expected value allowing for the diminished distance of traverse being in the latter case 0·0097.

(*g*) *The effect on the absorption rate of altering the dimensions of the apparatus.*

The effect of altering the size of the present apparatus while retaining its form is to cause an increase in the absorption rate proportional to the increase in the linear dimensions. The utilisation of this effect is limited by the fact that as we increase the surface area of the outer chamber the volume of fluid necessary to cover the surface will also increase. 5 or 10 cc. of fluid for analysis may be

Table III.

Area of floor of outer chamber cm.²	Area of floor of inner chamber cm.²	Height of inner chamber cm.	Height of outer chamber cm.	Distance of molecular passage* cm.	Absorption rate found (A_2 value = 2·30 $A_1 \times V$)	A_2 divided by the geom. mean of the areas multiplied by the distance of passage of mols.
66·8	43·5	1·0	2·0	13·9	0·091	0·023
22·7	16·6	0·6	1·6	8·6	0·064	0·028
43·1	14·8	0·8	1·3	9·6	0·062	0·024
7·9	17·8	0·5	1·0	7·0	0·047	0·027
16·7	9·6	0·5	1·0	6·9	0·044	0·024
25·6	8·6	0·85	1·65	8·3	0·037	0·021
8·5	1·76	0·80	1·40	4·4	0·025	0·028

Temperature = 23°.

* The mean distance of the molecular passage from the outer to inner chamber would be very difficult to state, but it has been taken as proportional to the total diameter *plus* the height of the outer chamber *minus* the fluid depths in the two chambers (the total diameter of the outer chamber is taken at the top and thus includes that of the inner chamber).

The absorption rates have been determined with pure ammonia solutions (N) in the outer chambers and N sulphuric acid in the inner. The figures in the last column show a fair constancy and are indicative of the general principles underlying the effect on the absorption rate of changes in the dimensions of the apparatus. Divergences from the mean value of these figures are largely attributable to irregular differences in the depth of the air passage between the inner and outer chambers.

It may be pointed out that the linear dimensions of the first and fifth type of unit investigated above are very nearly in the ratio of 2 to 1, and it will be observed that the absorption rates in the last column but one are also nearly in the same ratio, for these units.

The absorption rates given in the last column but one are the A_2 values representing the rate of absorption per minute per unit concentration in the outer chamber, or 2·30 $A_1 \times V$.

handled with apparatus of larger design. When the linear dimensions of the present apparatus are doubled and 10 cc. of fluid are used, the time for 99·5 % absorption (using saturated potassium carbonate) at 38° is 4 hours. With such an apparatus however the solid carbonate may be readily used and then the absorption time will reduce to approximately 1 hour at 38°.

When the linear dimensions of the present apparatus are altered in different proportions the following principles hold approximately.

(1) The absorption rate is proportional to the geometric mean of the surfaces of the inner and outer chambers.

(2) The absorption rate is inversely proportional to the mean distance of passage of the molecules.

These principles are illustrated in Table III where a certain number of apparatus of different design have been investigated.

3. Ammonia estimations.

Under this heading will be considered estimations carried out on standard solutions of ammonium sulphate with the apparatus of dimensions as described in Fig. 1. In regard to the actual estimation of ammonia three questions arise for consideration. Firstly, what degree of total absorption may be expected, secondly, what time will this absorption take, and finally, what degree of accuracy may be expected with each individual estimation. In the investigation of these questions we shall consider the analysis of 1 cc. volumes of the purest ammonium sulphate solutions free from pyridine salts. In the central chamber is placed 1 cc. of standard acid (from a 2 cc. standard burette) and 1 cc. of saturated potassium carbonate is added to the ammonium sulphate solution in the manner previously described.

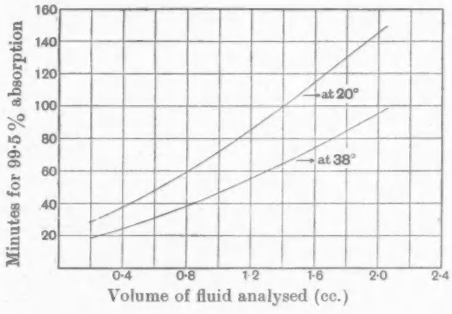

Fig. 3. The two curves give the minimum average times for 99·5 % absorption at 38° and 20° respectively. The times apply only when 1 cc. of saturated potassium carbonate is used in every instance, the figures for the abscissae giving the volume of fluid to which this alkali is added. In practice it is advisable to take 10–20 % in excess of these times. It is also to be noted that the curves apply to the standard apparatus as given in Fig. 1.

In regard to the total absorption, this value does not fall below 99·5 % when sufficient time is allowed for the absorption to take place. Fig. 3 gives the minimum times for 99·5 % absorption when 1 cc. of saturated potassium carbonate is used in every case but with different volumes of analysed fluid. The two curves are for room temperature (20°) and for 38°. In the actual carrying out of estimations it is advisable to allow an additional 10–20 % on the minimum time. From the curves it may be seen that the time for absorption using 1 cc. of fluid for analysis and at room temperature is 77 minutes, and at 38° it is 45 minutes. With smaller and larger quantities analysed these times vary correspondingly. The times for temperatures other than those given may be calculated on the basis of an alteration of 2·9 % per degree difference. If volumes of saturated potassium carbonate other than 1 cc. are used the absorption times may be calculated from the data previously given.

We may now consider the degree of accuracy to be expected for the individual estimation. With this object a large number of analyses were carried out on ammonium sulphate solutions of different strengths. In these estimations 1 cc. quantities of the ammonium sulphate solution were used with 1 cc. of standard sulphuric acid in the central chamber (equal in strength to the ammonium sulphate solution multiplied by a factor of 1·2). Deliveries of the ammonium sulphate and the acid as well as the final titrations were carried out with standard 2 cc. burettes. The results of a number of such estimations are given in Tables IV and V. It will be seen that the error of the individual estimation is low, the coefficient of variation being 0·50 for a determination of 1·4 mg. ammonia-nitrogen, and alters very little for a determination of 0·014 mg. The only limit to the delicacy of the estimation as we reduce the amount of ammonia for analysis is the error arising from the titration of 1 cc. of standard acid with carbon dioxide-free alkali.

Table IV.

No. in series	Ammonia absorption as cc. 0·1 *N* alkali	Ammonia absorbed as percentage of amount added to outer chamber	Ammonia absorbed expressed as mg. ammonia-nitrogen
1	1·970	98·5	1·38
2	1·990	99·5	1·39
3	1·980	99·0	1·40
4	1·984	99·2	1·39
5	1·980	99·0	1·39
6	2·050	102·5	1·43
7	2·000	100·0	1·40

The above 7 experiments are the first of a series of 48 and are typical of the results obtained by the method. In the experiments 1·40 mg. ammonia-nitrogen (as 1 cc. of 0·1 *N* ammonium sulphate) were added to the outer chamber, the free ammonia being liberated by 1 cc. of saturated potassium carbonate. The central chamber contained 1 cc. of 0·12 *N* sulphuric acid. After the addition of the carbonate the units were incubated for 1½ hours at 38°.

Table V.

Quantity of ammonia-nitrogen added to outer chamber mg.	Percentage "recovered" by absorption in inner chamber	Standard deviation of individual estimation as mg. ammonia-nitrogen	Coefficient of variation of individual estimation	Number of estimations
1·40	99·5	$7·0 \times 10^{-4}$	0·50	48
0·140	99·5	$6·7 \times 10^{-5}$	0·48	33
0·014	99·5	$1·0 \times 10^{-5}$	0·72	24

Conditions of analyses similar to those in previous table.

Where for very weak ammonia solutions a Nessler method is preferred this may be easily introduced by pipetting off the acid in the central chamber at the end of the absorption, and with suitable washings with small quantities of ammonia-free water transferring to a small volumetric flask, *etc.* Excellent results in the estimation of very minute quantities of ammonia may be obtained with very little practice.

Outline of a standard method for the analysis of micro-quantities of ammonia.

For the convenience of the reader a certain amount of the procedure already mentioned in the text will be briefly repeated with additional notes on the details.

A standard method may be described as follows. Into the central chamber of the absorption unit is run 1 cc. of standard acid of strength depending upon the

expected range of ammonia to be analysed. Into the outer chamber is placed a measured quantity of the fluid for examination. (It is advisable that this fluid should not exceed 2 cc. with the present apparatus.) The glass lid is suitably smeared with the fixative and placed in position on the unit. The unit is slightly tilted—most conveniently by resting the edge upon a spare lid—and the lid shifted so that a small opening is made into the outer chamber of sufficient size to allow the introduction of the tip of a pipette. Approximately 1 cc. of saturated potassium carbonate is quickly run in and the lid replaced. When introducing the carbonate no care need be taken as to the accuracy of the addition for an error of 10 % is of negligible effect on the absorption time. The carbonate is also best introduced from a pipette with a somewhat wide opening so that it quickly runs out without any blowing. The apparatus is now rotated gently for about 10 seconds to mix the fluids in the outer chamber and is then left aside at room temperature for 90–100 minutes, which will be sufficient time for 99·5 % absorption at ordinary room temperatures. Alternatively it may be incubated at 38° for 1 hour.

When the absorption is complete the lid is removed and the acid in the central chamber is titrated with standard alkali—which should be free from carbon dioxide when the amount of ammonia to be analysed falls below 0·14 mg. A very suitable indicator to use is that described by Tashiro [1922] for use with ammonia estimations, namely a mixture of methyl red and methylene blue in alcoholic solution (a modification of Tashiro's indicator is described below).

The following practical considerations may be found useful in carrying out the method.

(1) Any failure at first to get accurate results of the order described with minute quantities of ammonia will almost invariably be due to the inner chamber of the units being insufficiently clean in an acid-alkali sense. Where the quantity of ammonia analysed falls below 0·014 mg. particular attention must be given to this. Adequate cleanliness for all purposes is at the same time very easily obtained, and where the attendant is preparing a number of units the following routine of cleansing should be adopted. After use the units are washed out under the cold water tap. They are then washed in a stream of hot water—best directly under the hot water tap so that the fixative is quickly washed away: the washing is continued for some time after this. The units are now washed out with several changes of distilled water the fluid being run on to the centre of the inner chamber. As much water as possible is then shaken off the surface of the unit which is allowed to dry in air, the under surface being dried with a cloth—which of course should never be allowed to touch the inner surface. Alternatively, after washing as above the units may be stored in very weak acid (0·001 *N* approx.) containing a little of the indicator and then rinsed with distilled water, *etc.*, before use. This latter procedure is a very safe one for ensuring that no trace of alkali is present in the central chamber. It is not necessary that the units be perfectly dry before use, but it is advisable to remove any droplets from the top of the inner wall with filter-paper.

Where a Nessler modification is introduced the cleanliness of the inner chamber in the acid-alkali sense is not important.

(2) When working with the absorption times given above the potassium carbonate solution must be fully saturated and this of course is only certain when the solid remains in contact with the solution after adequate shaking. While introducing the carbonate solution into the units care must be taken against spattering, which however is very easily avoided, particularly since no blowing in of the solution is necessary if the tip of the carbonate pipette is a

little wide in bore; for this purpose a glass tube drawn out a little may be used and a mark made on the tube at approximately 1 cc. capacity.

(3) The formation of a dew on the surface of the lid after incubating is of no consequence. It may readily be shown that the relative rate of passage of any absorbed ammonia from such dew into the acid is much faster than from the fluid in the outer chamber.

(4) *The indicator.* A very sensitive indicator for ammonia estimations is a modification of the Tashiro indicator, *viz.* a mixture of methyl red and methylene blue prepared in the following way. A stock solution is first made of a pure alcoholic solution of methyl red (0·1 %). To 200 cc. of this are added 50 cc. of a similar solution of methylene blue (0·1 %). The purest reagents should be used. The solution is stored in a brown bottle. To prepare some indicator for use one volume is taken and 1 volume of alcohol added with 2 volumes of water. The fluid is now brought to the point of its maximum sensitivity—indicated by the point when its red colour has disappeared—by the addition of alkali (preferably free from carbon dioxide). A few drops of this indicator will be sufficient for use—or an exactly measured quantity when dealing with very weak solutions. The end-point is best judged as the point when there is no longer any trace of red in the fluid. Prepared and used in this way it is a most delicate indicator in its colour change in spite of the statement of Kolthoff [1931]. The raising of the p_H from 5·45 to 5·50 is detectable in the titration as a change from a just appreciable pink to a just appreciable green.

A method of preparing the indicator mixture used in this laboratory consists in making up the acid with 20 % alcohol and containing 1 cc. % of the above stock mixture—carefully neutralised in some water—which is then added before making the acid mixture up to the correct volume. In this way extremely weak acid-indicator mixtures will keep for months.

(5) *The standard acid.* This was prepared from *N* sulphuric acid by dilution. For very weak solutions carbon dioxide-free water was used. The indicator may be incorporated as above described.

(6) *The standard alkali.* When the titrating alkali is less than 0·01 *N* it is advisable to have it free from carbon dioxide. Such alkali may be prepared for the present purpose by any standard laboratory method. The very weak acids and alkalis are best stocked in paraffined bottles protected with soda-lime traps and provided with a siphon tube, the tubing being made of pyrex glass.

(7) *Carbon dioxide-free water.* This may be formed in quantity sufficiently free from carbon dioxide by evacuating a 6 litre container filled with distilled water for about 4 hours at the filter-pump and allowing the air to return through strong alkali, with suitable traps, *etc.* The container must be protected in use with a soda lime tube. The use of this water will be necessary only for the most minute analyses.

(8) *The fixative.* Where the units are left aside at room temperature, vaselin may be used. Where incubation is carried out at upwards of 38°, a mixture of vaselin and paraffin wax is advisable. This is formed by melting 3 parts vaselin with 1 part of paraffin wax (M.P. 55°) and allowing to cool.

Summary.

A very simple apparatus is described for the micro-estimation of certain volatile substances by simple diffusion, no aeration or distillation processes being involved. The absorption and estimation of ammonia with this apparatus is described. An absorption of 99·5 % of the ammonia content of 1 cc. of fluid is

established in less than 1½ hours at room temperature and in less than 1 hour at 38°. The various conditions influencing these absorption times are analysed in detail. The coefficient of variation of the individual estimation as obtained from a large number of analyses is 0·5 %, this accuracy of analysis being practically maintained with amounts as small as 0·014 mg. ammonia-nitrogen.

An advantage of the method is that a large number of estimations (upwards of 50) can be carried out simultaneously.

REFERENCES.

Abegg and Riesenfeld (1902). *Z. physikal. Chem.* **25**, 236.
Durig (1907). *Biochem. Z.* **4**, 69.
Gaus (1900). *Z. anorg. Chem.* **25**, 236.
Kolthoff (1931). Die Massanalyse. Zweiter Teil, 64. (Berlin.)
Mellor (1928). A comprehensive treatise on inorganic and theoretical chemistry, **8**, 195.
Michaelis (1929). Oxydations-Reduktions Potentiale, 75. (Berlin, Springer.)
Neuberg (1911). Der Harn, 91. (Berlin.)
Tashiro (1922). *Amer. J. Physiol.* **60**, 519.

LXII. AN ABSORPTION APPARATUS FOR THE MICRO-DETERMINATION OF CERTAIN VOLATILE SUBSTANCES.

II. THE DETERMINATION OF UREA AND AMMONIA IN BODY FLUIDS.

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(*Received January 9th, 1933.*)

The urea of blood.

The determination of urea is an obvious application of the previous method of determining ammonia [Conway and Byrne, 1933]. By the action of urease an equivalent quantity of ammonia is formed and determined as previously described. The ferment action is very conveniently carried out in the outer chamber of the unit. The volume of 0·2 cc. has been selected for illustration since it represents an amount of blood easily obtained from the finger or ear lobe, and the error of the individual estimation on standards of 50 mg./100 cc. is only 0·6 mg. (expressed as a standard deviation), which accuracy is easily attained.

Method for 0·2 cc. blood. Into the inner chamber of a clean dry unit is run 1 cc. of 0·00667 *N* sulphuric acid (Solution I). Into the outer chamber 0·2 cc. of the blood for analysis is introduced from an Ostwald pipette. This volume of blood for convenience is kept in the one region rather than distributed over the surface. (The pipette should be standardised for its exact blood delivery without washing.) 0·5 cc. of the urease-phosphate (Solution II) is now added from a pipette—being dropped on the fluid already in the outer chamber. The lid is placed on the unit, the fluid in the outer chamber is further mixed by rotation, and the apparatus is then set aside for 15 minutes at room temperature or 10 minutes at 38°. The unit is then slightly tilted, the glass lid is a little displaced to introduce the tip of a pipette and 1 cc. of saturated potassium carbonate run into the outer chamber, the lid being quickly replaced in position. The fluids in the outer chamber are now well mixed by rotation and then left aside at room temperature for 90 minutes or incubated for 1 hour at 38°. At the end of this time the acid in the central chamber is titrated with carbon dioxide-free alkali (0·00267 *N*) (Solution III) from a 2 cc. standard burette (provided with a soda-lime trap) using some of the special indicator described in the previous communication.

Blank estimations are carried out with the reagents, the ammonia of which should be practically negligible and may in fact be reduced to zero by a suitable manner of preparation. A few such blanks with the fresh reagents with subsequent occasional estimations will suffice. A blank with the blood *plus* carbonate alone is advisable in certain diseased conditions or where the blood has been long standing. When the blood is first drawn its ammonia content, as is well known, is normally quite negligible compared with the urea.

Calculation. The volume of titrating alkali in cc. corresponding to the urea-ammonia (after deducting the blank value) is multiplied by 40 to give the mg. urea per 100 cc. in the blood. This may be subsequently corrected for pipette and alkali factors. Where the titrating alkali does not give exactly 2·50 cc. for 1 cc. of the acid a factor must be correspondingly introduced—the acid being taken as the standard of reference. The estimations may be suitably controlled by using the above procedure on 0·05 % urea. A description of the solutions used follows.

Solution I. 0·00667 *N* sulphuric acid. This is prepared by running 33·33 cc. of *N* sulphuric acid into a 500 cc. flask and making up to volume with carbon dioxide-free water. This solution is diluted 10 times. The weak solution is best kept in a paraffined or pyrex bottle with soda-lime trap and siphon—the tubing being of pyrex glass. The solution should then keep indefinitely.

Solution II. 0·00267 *N* carbon dioxide-free sodium hydroxide. This may be prepared from carbon dioxide-free alkali prepared as indicated in the previous communication. The carbon dioxide-free alkali should be stored with the same precautions as the weak acid. The diluted solution should not be used for longer than a week.

Solution III. The urease-phosphate solution. A stock glycerol extract of urease is prepared as follows in the manner described by Schmidt [1928] with but little variation. 22 g. of finely powdered permutite are washed with 2 % acetic acid, which is then decanted off, the permutite being subsequently washed twice with distilled water. The permutite is mixed with 45 g. of finely powdered jack bean meal and 75 cc. of distilled water. These are shaken for about half an hour, and then 225 cc. of pure glycerol are added and mixed. The whole is filtered and the filtrate collected. To prepare the dilute solution for use, one volume of the glycerol extract is diluted with the addition of one volume of the phosphate solution to ten volumes. Sufficient of this urease-phosphate solution should be freshly prepared for each set of estimations.

Any other method—such as the alcoholic extract described by Folin [1923]—for preparing a urease solution will suffice, but the urease should be tested with the 0·05 % urea control before use.

The phosphate solution is made by dissolving 69 g. of NaH_2PO_4 and 179 g. of crystallised Na_2HPO_4 in 100 cc. of distilled water. It is stored in the refrigerator. At room temperature it tends to become mouldy.

As an illustration of the results obtained by the method Table I shows the first six analyses of a set of 19. Table II shows the results of some experiments on 0·2 cc. of different blood samples prepared from the one stock by adding 5 cc. of water or of a urea solution to 20 cc. of the blood and demonstrates the agreement of the "recovered" quantities with those added. The volume of acid used in the above estimations suffices for 100 mg. urea per 100 cc. If there is any reason to expect a greater urea content than this in the blood half the quantity of blood may be analysed.

Table I.

Number in series	Blank estimation *minus* titration figure in cc. of 0·00267 *N* alkali. (Factor of alkali = 1·012)	Estimated conc. of urea in the 0·2 cc. sample, corrected for alkali and pipette factors
1	1·225	50·1
2	1·215	49·7
3	1·200	49·1
4	1·215	49·7
5	1·225	50·3
6	1·230	50·3

The above 6 experiments are the first of a series of 19. In each 0·2 cc. of a standard urea solution (50 mg./100 cc.) was analysed. Factor of pipette = 0·990. Example of the calculation taking the first result: 1·225 cc. of alkali corrected for factor becomes 1·240. This multiplied by 40 gives 49·60 mg./100 cc. which corrected for the pipette factor gives 50·1 mg./100 cc.

Table II.

Blood sample	Urea conc. found mg./100 cc.	Urea added as mg./100 cc. of mixture	Urea "recovered" mg./100 cc.
I	26·8	00·0	—
II	46·4	20·0	19·6
III	58·6	30·0	31·8
IV	66·0	40·0	39·2
V	76·4	50·0	49·6

To make each blood sample 20 cc. of blood were taken from the same stock, and 5 cc. of water or of a standard urea solution were added, 0·2 cc. of each mixture being taken for analysis.

Use of Nessler's reagent for determination of the ammonia. The advantage of this modification is that it eliminates the use of weak acid and alkali solutions, as well as the use of standard 2 cc. burettes. Into the central chamber is run 1 cc. of $N/10$ or $N/100$ sulphuric acid, the remaining procedure up to the end of the absorption being the same as in the previous method.

The lid is now removed and the contents of the inner chamber are removed by a simple glass pipette with rubber teat and transferred to a 25 cc. volumetric flask, subsequently washing and transferring the washings in a similar manner. 5 cc. of Nessler's solution prepared according to the directions of Folin and Wu [1919] are added to the flask, and the mixture is shaken and made up to the 25 cc. mark. A standard is prepared for colorimetric comparison by making up 16·6 cc. of 0·1 N ammonium sulphate to a litre and taking 2 cc. of this with the addition of Nessler as before. Calculation of the result is as follows:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 50 = \text{mg. urea/100 cc. in blood.}$$

A blank is also carried out and the ammonia in it calculated in terms of urea percentage as in the above formula. This is subtracted from the first result.

Method for 1 cc. of a protein-free filtrate. In this section we may consider a Folin-Wu tungstate filtrate. The procedure is similar to the previous analysis except that 1 cc. of the filtrate from an Ostwald pipette is substituted for the 0·2 cc. of blood. The acid and alkali however should be half the strength of the reagents used in the first titration method with 0·2 cc. of blood. The time of the absorption after the addition of the carbonate may be taken as 2 hours at room temperature or $1\frac{1}{4}$ hours at 38°. The Nessler modification may be used as before, the standard being half the strength.

The method may be also extended to the analysis of 1 cc. of plasma with the present apparatus. The proteins present have no appreciable influence on the ammonia tension. With 1 cc. of whole blood the absorption is somewhat delayed by the formation of a scum after some time, due to the saturated carbonate addition.

In using 1 cc. of plasma the acid and alkali should be five times stronger than that previously described.

The urea of urine.

Method for 0·2 cc. of diluted urine. Seeing that in the analysis of 0·2 cc. of urea solutions of 50 mg./100 cc. the standard deviation is only 0·6 mg./100 cc., if no further accuracy is desired the urine may be diluted 50 times and estimated in the same way as blood. The dilution has the advantage that only one set of reagents need be used for urine and blood. With the diluted urine an additional

blank estimation is carried out to determine the small correction to be made for the ammonia in the urine. In this blank, if the ammonia content itself is not accurately required, the blank need only consist of the diluted urine and the potassium carbonate in the same volumes as above. Having determined the quantity of ammonia due to the urea alone (expressed as cc. of the titrating alkali) the calculation is the same as before—the result being finally multiplied by 50 for the dilution.

The analysis of 40 duplicate estimations of urine by this method showed an average difference in the duplicates of 0·5 mg./100 cc., that is the same average difference as shown in 40 duplicate blood analyses carried out in the same way.

As with blood the method may be extended to the analyses of larger volumes.

The ammonia of urine.

Method for 0·2 cc. of diluted urine. The urine is diluted four times and 0·2 cc. of the diluted urine analysed as in the preceding 0·2 cc. methods except that the urease is omitted. The absorption rate will here be faster owing to the omission of the urease. An hour at room temperature will suffice or about 40 minutes at 38°. A blank estimation may be carried out on the alkali and acid alone. In the calculation the result may be first expressed as though urea were estimated in the same way as in 0·2 cc. of blood. The calculated figure is multiplied by $\frac{17}{40}$ to reduce it to ammonia. The result is then multiplied by the dilution. The method as before can be extended to larger volumes of diluted or undiluted urine, using suitable strengths of absorbing acid and titrating alkali.

An advantage of the methods here described for the determination of urea in blood and urine and ammonia in urine on 0·2 cc. volumes is that the same acid and alkali may be used throughout.

Discussion.

In the analysis of 0·2 cc. of a standard urea solution—50 mg./100 cc.—by the method here described the error obtained expressed as a standard deviation was 0·6 mg. urea per 100 cc. or a coefficient of variation of 1·2. Similar results were obtained for the recovery of urea added to blood. These results are very easily reproducible. We may compare them with the Collip-Clark [1926] method for analysing the urea content of 5 cc. of blood-filtrate corresponding with 0·5 cc. of blood. This—by comparison—is a very elaborate method and was specially selected for the highest accuracy obtainable. After autoclaving to convert the urea to ammonia the latter is estimated by a Parnas-Pregl technique. With this technique the urea-ammonia will be present finally in about 10 cc. fluid.

Using methyl red as indicator, the error of titration as given by Pregl [1930] is $\pm$ 0·01 cc. of 0·01 N alkali. If we take as an example a blood-urea concentration of 50 mg./100 cc. the amount of 0·01 N alkali corresponding to the urea-ammonia analysed is 0·83 cc. The error therefore expressed as a percentage of the mean is 1·2. This arises from the titration alone without reckoning any other source. If we consider Pregl to have stated the titration error as a standard deviation it corresponds to the total error arising in the estimate of 0·2 cc. of blood by the method described in this communication. If however we take Pregl's figure as some practical statement of the range within which the observations fall, this range may be taken as $\pm$ 2 $\times$ the standard deviation. (It is scarcely necessary to point out that all such statements of error should be given definitely as standard deviations or coefficients of variation.) With the latter

assumption the standard deviation of titration of the individual estimation by the Collip-Clark method expressed as a coefficient of variation is ± 0·6 (where the standard analysed is 50 mg. urea per 100 cc.). The additional errors arising from the measurement of volumes, distillation, autoclaving *etc.*, must bring the final result to within ± 1·0 or what is practically equivalent to the error of the method here described. The latter however is carried out on two-fifths of the quantity of blood and has the advantage of an extreme simplicity of procedure and apparatus.

The present micro-method for urea in 0·2 cc. of blood compares very favourably with the comparatively macro-method of Van Slyke and Cullen [1914] in which 3 cc. of blood are used and seems to give but the same percentage error. As an illustration we may take the results of Addis and Watanabe [1916] who carried out a large number of estimations by the Van Slyke and Cullen method. They say that in 90 duplicate estimations of blood-urea the "average error" was 1·5 mg./100 cc. In a footnote to their paper however they state that this average error of the duplicates was subsequently reduced to 0·5 mg. which we may suppose to be the least error obtainable. In 7 duplicate blood estimations carried out personally by the author using 0·2 cc. of blood, with the present method the average error was 0·8 mg. urea per 100 cc. In 40 duplicate estimates carried out by a skilled attendant the "average error" of the duplicates was 0·52 mg. urea per 100 cc. The results therefore show the same degree of accuracy using 0·2 cc. of blood as in the Van Slyke and Cullen method using 3 cc., with the advantage of a much greater simplicity of technique and the possibility of carrying out larger numbers of estimates at the one time.

Summary.

Methods have been described for the estimation of urea in blood and urine and for the estimation of the ammonia in urine. The analyses were conducted on 0·2 cc. volumes, but larger volumes may be used. The error of estimation of the urea in 0·2 cc. of blood expressed as a standard deviation is 0·6 mg./100 cc. The technique is an obvious extension of that used in the previous communication for the estimation of ammonia, the urea being converted into equivalent quantities of ammonia by the action of urease obtained from the jack bean as a glycerol extract. Large numbers of estimations may be carried out at the one time and the percentage error of the urea estimation on 0·2 cc. blood does not exceed that found in the Van Slyke and Cullen method using fifteen times the quantity of blood nor, apparently, that found in the Collip-Clark method using two and a half times the volume.

REFERENCES.

Addis and Watanabe (1916). *J. Biol. Chem.* **24**, 203.
Collip and Clark (1926). *J. Biol. Chem.* **67**, 679.
Conway and Byrne (1933). *Biochem. J.* **27**, 419.
Folin (1923). Laboratory manual of biological chemistry, 3rd edition. (New York and London.)
—— and Wu (1919). *J. Biol. Chem.* **38**, 81.
Pregl (1930). Quantitative organic microanalysis, 2nd English edit., 116. (London.)
Schmidt (1928). *J. Biol. Chem.* **78**, 54.
Van Slyke and Cullen (1914). *J. Biol. Chem.* **19**, 211.

LXIII. A NOTE ON THE DESORPTION OF *n*-BUTYLAMINE FROM CHARCOAL.

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(*Received January 26th, 1933.*)

Although the desorption of substances from charcoal and other adsorbents has become an essential step in many important chemical processes, notably the isolation of substances of biological interest, the physical chemistry of the recovery process has received very little attention. In the field of vitamin chemistry, for example, the use of adsorbents such as charcoal [Kinnersley and Peters, 1927] and fuller's earth [Seidell, 1916] has led to the concentration of the curative substances to a very high degree. The conditions under which the recovery process has been carried out seem in general to have been determined by the use of excess of the solvent from which adsorption took place, or, preferably, of a solvent in which the adsorbed substance is more soluble.

Approaching the problem from the physico-chemical standpoint, Scharrer and Schwaibold [1929] have found that potassium iodide adsorbed on various earths is desorbed only partially, even by continual washing with water; soluble iodides and iodates behave similarly. Kōda [1928] has investigated the desorption of potassium iodide by water from blood charcoal and *bolus alba*. He finds that desorption proceeds more rapidly than the corresponding adsorption process, and that equilibrium is reached more rapidly with increasing temperature.

The present authors recently communicated to the Society a preliminary review of the problem [Vallender and Phelps, 1931] which has aroused sufficient interest and discussion to lead them to a more elaborate investigation.

Purified charcoal was prepared by the method already described [Phelps and Peters, 1929]. The charcoal used in the present experiments was, however, heated *in vacuo* for a considerably greater period and was presumably more completely degassed. There is strong evidence that *n*-butylamine adsorbed on purified charcoal is in the form of un-ionised molecules [Phelps and Peters, 1929]. It must be pointed out that this is only true of acid-treated charcoal if the base is adsorbed from alkaline solution. In the presence of butylammonium salts a slight amount of polar adsorption probably occurs [Phelps, 1931].

The sample of *n*-butylamine used was purified by redistillation. The amine was estimated by steam distillation in a micro-Kjeldahl apparatus, the base being trapped in $N/70$ sulphuric acid, which was back-titrated with equivalent alkali. With the concentrations used in this work the error of such determinations certainly did not exceed 0·5 %.

Experimental.

Technique of the adsorption and desorption experiments.

Samples of 20 cc. of 0·2 % *n*-butylamine solution were shaken with 200 mg. of purified charcoal for periods of from 12 to 16 hours. The charcoal was then filtered off and dried as rapidly and completely as possible by pressing between filter-papers. It was found that Whatman No. 40 filter circles did not adsorb

n-butylamine to any measurable extent. The dried charcoal was then immediately suspended in the desorbing liquid. Each 200 mg. sample of charcoal was extracted with 20 cc. of liquid. This technique is open to obvious objections from a purely theoretical standpoint, but since it follows the methods generally employed in large scale preparations by selective adsorption, it was chosen in this case as likely to yield results of the greatest practical value to the biochemist.

A number of such suspensions were then shaken in a thermostat, each for a definite time, after which the charcoal was removed by filtration and the concentration of *n*-butylamine in the filtrates determined. The filtrates from the original adsorption experiments were also analysed in order to check the amount of base adsorbed by the charcoal. Experiments in which the original adsorption did not agree closely with the mean value were rejected. This inevitably varied slightly (not more than 5 %) from one sample of charcoal to another owing to slight differences in the state of activation or fineness of division. Between these narrow limits, however, the relative amount of base desorbed seemed independent of the original adsorption.

Results.

Initially three series of experiments were carried out, the desorption of *n*-butylamine by pure water being studied at 0°, 15° and 25° respectively. In all the adsorption and desorption experiments the proportion of charcoal to water or solution was kept at 10 mg. to 1 cc. The experimentally determined values of the amount of base recovered from the charcoal after various periods of time can, therefore, be used to construct curves showing the actual course of the recovery process at the temperatures specified.

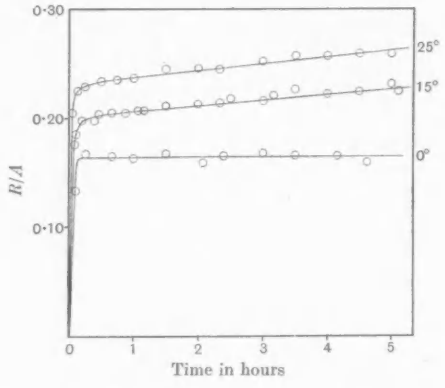

Fig. 1. Curves representing the progress of the desorption of *n*-butylamine from charcoal by pure water at 0°, 15° and 25°. R/A is the fraction of the total base originally adsorbed which is desorbed in a given time.

The corresponding values of the ratio of the amount of base desorbed to the amount originally adsorbed on the charcoal have been calculated, since the value of this ratio (R/A) is perhaps of more interest from the practical standpoint than the absolute amount desorbed. For this reason the progress of the desorption is represented graphically by plotting values of the ratio (R/A) against time.

Fig. 1 represents by three curves the progress of the desorption of *n*-butylamine by water at 0°, 15° and 25° respectively. Consideration of these curves makes clear certain characteristics of the process of desorption. In the first place, it is a two-stage process. There is first a very rapid release of about one-fifth of the adsorbed base from the charcoal. The velocity of this process is such that its temperature coefficient cannot be established by the experimental technique employed in this work. It is clear, however, that the amount of base desorbed in this process increases with increase of temperature.

This rapid process is extremely brief, and is succeeded by a phase of slow desorption, which continues with constant velocity up to 5 hours at least. In this process about one-fortieth of the base originally adsorbed is released in 5 hours at 25°. The secondary phase of slow desorption has a considerable temperature coefficient and cannot be observed to proceed at all at 0°. This raises the interesting practical point that in the recovery of substances from charcoal at 0° (a not unusual proceeding in the isolation of unstable organic substances) there is nothing to be gained by more than the briefest treatment with any one sample of solvent.

It has been suggested [Kōda, 1928] that the process of desorption is a simple reversal of adsorption. Preliminary determinations of the velocity of adsorption of *n*-butylamine from 20 cc. of 0·2 % solution by 200 mg. of charcoal indicate that the process resembles that of desorption in that it consists of a rapid and a slow phase, but that at 15° the velocity of the former is greater, and that of the latter considerably less, than the corresponding velocities observed in the case of desorption.

McBain [1919] has interpreted the two-stage nature of the process of adsorption by charcoal as a simple covering of the external surface (adsorption), which very rapidly reaches equilibrium, and which is followed by a gradual infiltration of the adsorbate to capillaries and internal surfaces in the charcoal (absorption). This secondary process may continue for years. In the same way the distinction between the rapid and subsequent gradual processes of desorption is so clear that it might be thought to indicate the successive breaking of two different adsorption linkages; the primary rapid phase being regarded as the removal of molecules from the surface of the charcoal (McBain's adsorbed layer), while the secondary process would represent the migration of molecules from capillaries in the body of the adsorbent (McBain's absorbed substance). If the primary rapid process of desorption is regarded as representing the complete removal of the surface adsorbed layer, one would expect that, were desorption stopped immediately the rapid process was at an end, and the adsorbent dried and once more extracted with water, desorption would then proceed immediately with a velocity approximating to the velocity of the slower phase of the first desorption, since all the molecules susceptible of rapid desorption should have been removed already. This was not the case. Charcoal which had been desorbed for 1 hour (after which time the rapid initial process might reasonably be regarded as concluded) was dried and again extracted with 20 cc. of water. Again there was a rapid release of butylamine for a short time (during which about one-seventh of the residual adsorbed base was extracted) and subsequently a phase of slow desorption, which proceeded a little more slowly than does the corresponding process in the initial extraction of the charcoal. Table I shows the relative amounts of base desorbed from charcoal during the first and second extractions after 1 and 6 hours respectively. In this case the ratio R/A represents, as before, that fraction of the adsorbate originally present, which is recovered in the given time, while the ratio R_1/A_1 represents the proportion of

the adsorbate remaining from the first extraction which is desorbed after a given time during the second extraction process.

Table I.

Time of desorption (hours)	R/A	R_1/A_1
1	0·206	0·147
6	0·228	0·165

From these results it would appear that the primary rapid process of desorption must be regarded as reaching a state of kinetic equilibrium, determined by the concentrations of adsorbate in the solid and liquid phases. The secondary process of removal of adsorbate from the interior of the adsorbent must, in this case, be assumed to proceed at a rate which is not considerably influenced by the concentration gradient at the interface over wide limits.

In view of the fact that *n*-butylamine is a surface-active substance (approximate measurement indicates that a 0·1 % solution of *n*-butylamine lowers the surface tension of water by about 2 dynes per cm.), an alternative explanation of the results suggests itself. Butylamine in solution will, by Gibbs's theory, tend to be concentrated at the boundaries of the liquid phase. When charcoal on which the base is adsorbed is brought into contact with water, butylamine will be discharged into the water phase very rapidly. This will lower the surface tension at the charcoal-water interface, as a result of which butylamine molecules will be concentrated in the interface and the presence of this layer of relatively concentrated butylamine solution will hinder the migration of butylamine molecules from the surface. It is known that the establishment of surface tension equilibria at fresh surfaces is a very rapid process [Hiss, 1913: a dissertation quoted by Freundlich, *Colloid and Capillary Chemistry*, 3rd English edition, p. 51; the present authors have had no opportunity of seeing the original paper], and it is at least probable that the establishment of equilibrium at a charcoal-water interface is also rapid. It might be assumed, therefore, that the rapid fall of the rate of desorption to a constant low value is due to the setting up in the interface of a layer of concentrated solution, through which molecules leaving the surface migrate only with difficulty.

Desorption by alcoholic solutions.

In order to decide which of these two assumptions was the more probable, it was necessary to study the desorption of *n*-butylamine from charcoal by a solvent in which the base was not surface-active. In this case there could be no interface concentration of the adsorbate, and, if the second of the two hypotheses advanced above is correct, the process of desorption should proceed in one stage to completion or to kinetic equilibrium. If, on the other hand, desorption involves the successive breaking of two different types of adsorption linkages, the break in the desorption velocity curve might be expected in the absence of surface tension effects.

It has been found by other workers that an equi-volume mixture of water and ethyl alcohol is a very efficient desorbing agent, and this fact has been of great practical advantage in the concentration of vitamins of the "B" group [Kinnersley and Peters, 1927]. Approximate measurement showed that the surface tension of an equi-volume mixture of ethyl alcohol and water was not lowered to a detectable extent by 0·1 % of *n*-butylamine. Charcoal containing *n*-butylamine adsorbed from water solution was therefore extracted with an

equi-volume alcohol-water mixture, and the course of the desorption followed in three series of experiments at 0°, 14° and 25° respectively. The experimental technique exactly followed that previously described. The results are shown in Fig. 2 in which the values of the fraction R/A are plotted against time. The dotted line in the diagram represents the velocity of desorption by water at 25° plotted on the same scale.

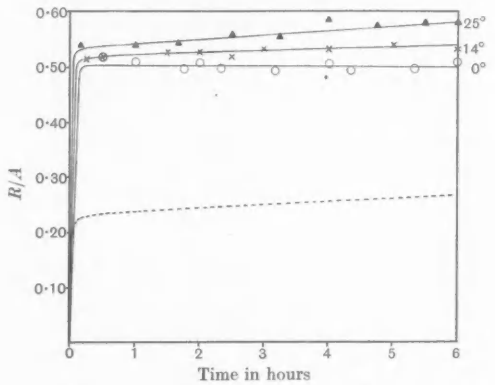

Fig. 2. Curves representing the progress of the desorption of *n*-butylamine from charcoal by equi-volume alcohol-water mixtures at 0°, 14° and 25°. The dotted curve shows the desorption of *n*-butylamine by pure water plotted to the same scale.

It will be seen that the two-stage nature of the desorption process is even more marked than in the case of desorption by water. During the primary rapid phase a considerably greater fraction (approximately one-half) of the adsorbed substance is discharged, but the duration of the rapid phase is roughly the same. The kinetics of the slower phase of desorption by alcohol-water mixtures show a very striking resemblance to the kinetics of the same stage of water desorption. In both cases the slower phase of desorption does not proceed at all at 0°, and the influence of increasing temperature is in either case the same.

Discussion of results.

All the results described seem consistent with the view that the process of desorption from charcoal by solvents involves the breaking of two distinct types of adsorption linkage. Such a scheme is consistent with McBain's view of sorption as involving both the rapid covering of external surfaces, "adsorption," and a slower process of infiltration to capillaries and internal surfaces of the solid phase, "absorption." The primary stage in the desorption of *n*-butylamine is rapid, and is therefore to be regarded as the removal of base "adsorbed" at the external surface of the charcoal. During this process water desorbs considerably less butylamine than does an equi-volume mixture of water and alcohol under the same conditions. This is in all probability due to the concentration of the base in the charcoal/water interface by surface tension effects in the former case. On the other hand, butylamine does not lower the surface tension of equi-volume alcohol-water mixtures, and there will therefore be no

tendency for the base to be concentrated at the interface between this solvent and charcoal.

In either case the primary rapid stage of desorption may be regarded as having reached equilibrium in 10 minutes at most. It is succeeded by very slow desorption, which proceeds at the same rate and has the same temperature coefficient both when water and alcohol-water mixtures are used as desorbing agents. In neither case does this process proceed at a measurable velocity at 0°. Since the rate of this slow secondary process is unaffected by surface tension effects, or, over a wide range, by changes in the concentration of the adsorbate in the liquid phase, and since, moreover, it continues constant for a long period, the secondary process must represent the dissociation of an adsorption complex which is re-formed only very slowly. This may be the reversal of the process leading, in McBain's view, to "absorption." There is nothing in the results of the present work which leads inevitably to the conclusion that the *n*-butylamine extracted in the secondary slow desorption process has been "absorbed" in capillaries or internal surfaces in the charcoal, but the existence of two types of adsorption complex, one much more stable than the other, is consistent with such a view.

The technique of desorption as a practical process in the isolation of chemical compounds may be considered briefly in the light of these results. In general, the empirical methods already described are justified. It would seem that the most complete desorption is to be attained by successive extraction of the adsorbent with different samples of solvent for short periods of time; more especially is this true if desorption is carried out at temperatures of 0° or lower. In the desorption of surface-active compounds (into which class many substances of biological importance undoubtedly fall), the use of a solvent such as an equi-volume alcohol-water mixture is to be recommended, unless the insolubility, or instability of the compound in alcoholic solution, renders this inadmissible.

Summary.

The desorption of *n*-butylamine from charcoal by pure water and by an equi-volume mixture of water and alcohol has been investigated.

Desorption is found to be a two-stage process in either case. There is first a period of very rapid desorption during which alcohol-water mixtures extract about twice as much adsorbed base as will pure water. The rapid phase of desorption attains kinetic equilibrium in a few minutes and is succeeded by a much slower process of desorption which continues with constant velocity for many hours. The slower process is considerably accelerated by rise of temperature, and at 0° is immeasurably slow. The velocities and temperature coefficients of the slow secondary desorption process are the same in pure water and in alcohol-water mixtures.

It is concluded that two different types of adsorption complex exist in the charcoal, one being far more stable than the other.

The thanks of one of the authors (H. J. P.) is due to the Royal Society for a grant whereby the expenses of this investigation have been in part defrayed.

His sincere thanks are due also to Prof. R. A. Peters and to Mr C. N. Hinshelwood with whom he has discussed the practical and theoretical aspects of this problem.

REFERENCES.

Hiss (1913). Über die zeitliche Änderung reiner Flüssigkeitsoberflächen. (Dissertation, Heidelberg.)
Kinnersley and Peters (1927). *Biochem. J.* **21**, 777.
Kōda (1928). *Acta Schol. Med. Univ. Imp. Kioto*, **10**, 1.
McBain (1919). *Trans. Far. Soc.* **14**, 202.
Phelps (1931). *Proc. Roy. Soc. Lond.* A **133**, 155
—— and Peters (1929). *Proc. Roy. Soc. Lond.* A **124**, 584.
Scharrar and Schwaibold (1929). *Z. anorg. Chem.* **182**, 193.
Seidell (1916). *U.S. Public Health Rep.* **33**, 314.
Vallender and Phelps (1931). *Chem. Ind.* **50**, 443.

LXIV. AN IMPROVED METHOD FOR THE PREPARATION OF DIHYDROXYACETONE.

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(*Received Febraary 18th, 1933.*)

Bacterial fermentation of glycerol with the production of dihydroxyacetone was first observed by Bertrand [1898], who isolated an organism—the so-called sorbose bacillus[1]—able to oxidise certain secondary alcohols to the corresponding ketone sugars. An organism biochemically related to the sorbose bacillus, which oxidises glycerol almost quantitatively to dihydroxyacetone, has been described by Virtanen and Bärlund [1926]. Similar organisms have later been isolated by Kluyver and Bernhauer.

The isolation of dihydroxyacetone from the metabolism solution was first described by Bertrand [1904]. He isolated the main portion of the triose by direct crystallisation, after removing the impurities by precipitation with alcohol and ether and evaporating the solution *in vacuo*. To increase the yield the triose present in the mother-liquor was converted into the bisulphite compound and liberated by sulphuric acid. A yield of 25 % from glycerol could thus be obtained.

This old method of isolation does not, however, lead to pure dihydroxyacetone, since it is not possible to free the product from traces of unfermented glycerol and other impurities. The product is consequently of a yellowish colour and has a tendency to remain in the syrupy form. To obtain pure dihydroxyacetone this first product must either be recrystallised or distilled in a high vacuum, when in both cases very great losses always occur.

For these reasons we have worked out a simple method for the preparation of dihydroxyacetone in a pure condition. The chief feature of our method is that the total amount of the ketone formed by the fermentation process is converted into the bisulphite compound, the latter being very carefully washed with alcohol to remove all traces of glycerol. From this compound the triose is liberated by sulphuric acid, and the salts are separated by repeated precipitation with alcohol and ether.

Experimental.

Organism and culture medium used.

The strain of *B. dioxyacetonicum* used in our experiments in 1926 has unfortunately died out. The present investigations were carried out with the *Acetobacter suboxydans* kindly sent to us by Prof. Kluyver of Delft. The culture medium was a 4–5·5 % solution of glycerol in a 3 % extract of yeast. The optimum concentration of glycerol appeared to be 5–5·5 %. The volume of the medium was in all experiments 1000 cc., in 6 litre conical flasks. The reaction of

[1] The sorbose bacillus was later found to be identical with the well-known acetic acid bacterium *B. xylinum* [Emmerling, 1899].

the medium was adjusted to p_H 5·2, after which the flasks were autoclaved. All inoculations were made with pure cultures of *A. suboxydans* grown at 20° for 4 days. The flasks were maintained at a temperature of 20° (± 1°) for about 14 days during which time the fermentation came to an end[1]. This was ascertained by taking small samples of the culture fluid (under aseptic conditions) and following the increase of reducing power. The dihydroxyacetone content of the flasks was determined and calculated according to Fürth and Marian [1926]. Table I shows the results of an experiment with 5 flasks, each containing 53 g. of glycerol in 1000 cc. culture medium with an initial reaction of p_H 5·22. The fermentation came to an end after 14 days.

Table I.

No.	p_H	cc. 0·153 $KMnO_4$ per 2·0 cc. metabolism solution	Dihydroxyacetone g. in 1000 cc.	Dihydroxyacetone % from glycerol
1	2·80	12·2	45·35	85·6
2	Not determined	12·5	46·65	88·0
3	2·88	13·0	48·75	92·0
4	Not determined	12·3	45·85	86·6
5	„	12·2	45·35	85·6

From 265 g. glycerol 231·95 g. dihydroxyacetone were thus formed, an average yield of 87·5 %.

The isolation of dihydroxyacetone.

In order to isolate the dihydroxyacetone the united metabolism solutions were centrifuged clear and evaporated to a syrup in large porcelain dishes at 37° in a powerful current of air from an electric fan. The syrup was then dissolved in alcohol and ether (100 cc. alcohol and 40 cc. ether for each 100 cc. of syrup) to precipitate the impurities, and the alcohol-ether solution was again concentrated to a syrup. During this procedure small amounts of dihydroxyacetone are lost and the triose content of the syrup must consequently be redetermined before further operations.

The syrup was next neutralised with sodium hydroxide, and an amount of $NaHSO_3$ corresponding to the amount of triose present (1·16 parts of $NaHSO_3$ for 1 part dihydroxyacetone) was added as a saturated hot solution. The mixture was allowed to stand for half an hour and then cooled with an ice-NaCl mixture to about 0–4°. Cold alcohol was added to complete the precipitation of the triose-bisulphite compound.

The crystalline mass was filtered off and thoroughly washed with alcohol. The yield is quantitative.

The bisulphite compound was dissolved in a small quantity of water and the corresponding amount of 15 % sulphuric acid added. The reaction mixture was held at a temperature of 50–60° and a vigorous air-current drawn through the solution until SO_2 was completely removed. The solution was then neutralised with 2*N* NaOH and concentrated at 37° until crystals of Na_2SO_4 began to separate. The precipitation of inorganic salts was completed by addition of alcohol, and the filtrate and washings (alcohol) were evaporated to a syrup. The small amounts of Na_2SO_4 still present in the syrup were removed by a further precipitation with alcohol and eventually with ether, and the clear filtrate was

[1] In this connection it may be mentioned that the acidity of the metabolism solution was invariably higher after completed fermentation than at the beginning of the experiment, amounting to about p_H 3. The cause of this increase of acidity was not further investigated.

again concentrated to a syrup. The last traces of water and alcohol were removed by heating the syrup *in vacuo* to 50°.

The resulting slightly yellowish syrup was kept *in vacuo* in an ice-chest and crystallised within a few days to a hard white crystalline mass, which was ground to a powder and thoroughly washed with cold absolute alcohol. The resulting product was a pure white crystalline powder and consisted of very pure dihydroxyacetone (M.P. 74–75°).

By careful working, a yield of 80 % from the triose formed could be obtained in crystalline form. The final washing with cold absolute alcohol reduced this yield by about 50 %, the other half being dissolved by the alcohol, from which it could not be obtained in crystalline form. With a fermentation yield of 85 %, about 40 % calculated on the triose formed, or about 35 % on the glycerol used, was thus obtained as pure dihydroxyacetone.

REFERENCES.

Bertrand (1898). *Compt. Rend. Acad. Sci.* **126**, 842, 984.
—— (1904). *Ann. Chim.* (8) **3**, 253.
Emmerling (1899). *Ber. deutsch. chem. Ges.* **32**, 541.
Fürth and Marian (1926). *Biochem. Z.* **167**, 135.
Virtanen and Bärlund (1926). *Biochem. Z.* **169**, 169.

LXV. THE INFLUENCE OF THE LENGTH OF THE TEST PERIOD ON THE ACCURACY OBTAINABLE IN A VITAMIN A TEST.

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(*Received February 23rd, 1933.*)

Sherman and Batchelder [1931] have claimed that, in a vitamin A test, "a decrease of 25 per cent. (or an increase of 33 per cent.) in the amount of vitamin A given is undoubtedly measurable by the method here used, when nine or more carefully standardised experimental animals are employed at each level of feeding." This degree of accuracy is greater than that claimed for tests carried out in our own laboratory [Coward, 1932]. A comparison of Sherman's method with our own has revealed several points of interest, some of which will be dealt with later. One of them, however, it was possible to investigate at once. The length of Sherman's test period (*i.e.* the period during which the dose of vitamin A was given daily after the rat had ceased to grow on a diet deficient in vitamin A) is 8 weeks, whereas our own test period is 5 weeks. It seemed at least possible that the longer the test period, the greater would be the degree of accuracy obtained. From the examination of our own experiments, it is evident that this is true only to the following extent: the degree of accuracy is much greater in a 2 weeks' test than in a 1 week's test; it is also greater in a 3 weeks' test than in a 2 weeks' test: as the test period is prolonged beyond 3 weeks, the accuracy still increases, but so very slowly that it is clearly evident that it is not worth while to carry on a vitamin A test longer than 3 weeks.

The method of calculation.

Coward *et al.* [1930] published a curve of response of groups of rats to graded doses of vitamin A. Rats which had ceased to grow on a diet deficient in vitamin A were divided into groups of about 30 rats each. Each rat of a group was given the same daily dose of cod-liver oil for 5 weeks. The doses of the different groups ranged from 0·25 to 7·5 mg. The mean increases in weight in 5 weeks of the different groups were plotted against the dose of cod-liver oil given. The curve proved to be logarithmic. Later [1931] the results from the bucks and does of these groups were averaged separately, and again logarithmic curves of response were obtained.

For the purpose of the present calculation, the mean increases in weight of these same rats at the end of 1, 2, 3 and 4 weeks have been calculated and plotted against the doses of cod-liver oil given. Again logarithmic curves have been obtained (Fig. 1).

Coward [1932] determined the degree of accuracy obtainable by the use of 10 animals in a 5 weeks' test carried out under the conditions of the experiment

for the making of the curves of response. Up to that time, 108 tests had been made in this laboratory, partly on different substances and partly on different doses of the same substance. The mean variance of the test was determined from the variances of the average increases in weight in 5 weeks. Bucks and does were determined separately. From this were calculated the standard deviation of the increase in weight, the standard error when 10 rats were used in a test, and, by reference to the curves of response, the range of dose of vitamin A corresponding to the range of mean increase. Thus it was found that, by the use of 10 bucks, a result would be obtained 21 out of 22 times within 35 % below (or 53 % above) the true result. This corresponds to a probable error (1 out of 2 times) of about 13 % below (or 15 % above) the true value. It was found that about twice as many does as bucks were required for any particular degree of accuracy.

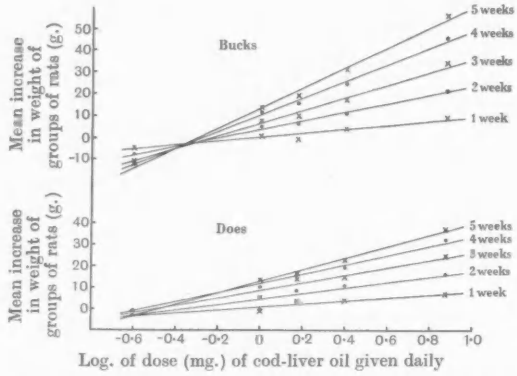

Fig. 1. Curves of response of different groups of rats to graded doses of vitamin A. Different groups of about 15 rats each, which had ceased to grow on a diet deficient in vitamin A, were given different daily doses of a particular sample of cod-liver oil, every rat in a group being given the same dose. The mean increases in weight of the groups of rats in 1, 2, 3, 4 and 5 weeks have been plotted against the logarithms of the doses of cod-liver oil given and regression lines have been drawn for each set of points.

In the same way, the degrees of accuracy obtainable in tests of 1, 2, 3 and 4 weeks' duration have been calculated. Up to date 201 tests, including the 108 already mentioned, have been carried out in the last 3 years in this laboratory under conditions of diet, housing and care of animals as nearly uniform as possible. The mean variances of the increases in weight after 1, 2, 3, 4 and 5 weeks respectively have been calculated. Every rat alive at the end of each week has been included in the calculation. No rat has been excluded for any reason whatever. The probable standard error $\left(\frac{2}{3}\cdot\frac{\sigma}{\sqrt{n}}\right)$ of the mean increases in weight of groups of 10 rats at the end of 1, 2, 3, 4 and 5 weeks respectively were calculated. By means of the corresponding curves of response for 1, 2, 3, 4 and 5 weeks, the range of dose corresponding to the range of mean increase $\pm\frac{2}{3}\epsilon$ was calculated, and from this the probable error of the result was expressed as a percentage of the result found. Thus the probable errors of results obtained by carrying on tests for 1, 2, 3, 4 and 5 weeks respectively could be compared.

DETAILS OF THE CALCULATION.

A. *The mean variance of the increase in weight.*

The mean variance of the increase in weight was calculated from the formula $\sigma^2 = \frac{\Sigma d^2}{N-m}$ where Σd^2 = the sum of the squares of the deviations from the means of the respective tests;

N = the total number of observations (*i.e.* the number of rats);

m = the number of means (*i.e.* the number of tests).

Out of 1129 bucks alive at the end of 1 week in these tests, 960 were alive at the end of 5 weeks. (It must be remembered that the tests included several in which the dose proved to be too low in vitamin A even to keep the rat alive for 5 weeks). Out of 1282 does alive at the end of 1 week, 1110 were alive at the end of 5 weeks. Most of the tests were made on groups of rats consisting of both bucks and does, so that actually there were 191 groups of bucks consisting of at least 2 animals at the end of the first week, and 177 such groups at the end of 5 weeks. Similarly there were 199 groups of does at the end of 1 week and 186 groups at the end of 5 weeks. The calculations of the mean variance and standard deviation of the increase in weight at the end of 1, 2, 3, 4 and 5 weeks, are given in Table I.

Table I. *The mean variance (σ^2) and standard deviation (σ) of the increase in weight in 1, 2, 3, 4 and 5 weeks respectively of male and female rats in a vitamin A test (resumption of growth after cessation on a vitamin A-deficient diet).*

Duration of test, weeks	Mean variance (σ^2)		Standard deviation (σ)	
	Bucks	Does	Bucks	Does
1	46·49	31·27	6·82	5·59
2	93·19	64·73	9·65	8·05
3	143·09	96·29	11·96	9·81
4	184·63	123·35	13·59	11·11
5	236·17	142·35	15·37	11·93

The mean variances are plotted as a graph in Fig. 2. It is evident that the curve of the bucks relating the mean variance to the duration of the test is a straight line through the origin. The curve of the does is a straight line through the origin at first, but deviates from the straight slightly at the third week and more and more at the fourth and fifth weeks, though the mean variance still increases with time within the limits (5 weeks) of this experiment.

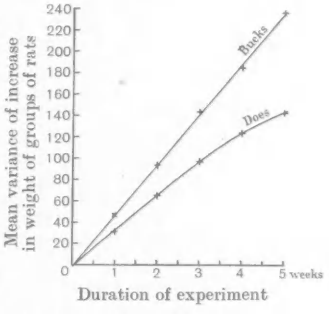

Fig. 2. The relation of the mean variance of the increase in weight of about 1100 bucks and 1300 does to the duration of the test period of a vitamin A estimation.

This conclusion is contrary to that of Norris and Church [1932], who thought that the differences in response of different rats to the same dose of vitamin A became less as the test extended from 1 to 5 weeks. In a few of our tests this has happened also, but it is clear from Table I and Fig. 2 that, in general, the variance and standard deviation of the increase in weight increase with the length of time of the test.

The fact that the mean variance of the does does not increase so rapidly

towards the end of the 5 weeks' test as at the beginning cannot be accounted for by the fact that some of the rapidly-growing animals were approaching maturity, growing more slowly, and were thus lessening the differences in increase in weight, for it was shown by Coward [1932] that the standard deviation of the mean increase did not vary with variations in the mean increase (*i.e.* within the limits of this test).

The standard deviations of both bucks and does as determined from these 201 tests are slightly higher than those determined from the first 108 tests. (Earlier tests: bucks 14·72, does 11·02; whole number of tests: bucks 15·37, does 11·93.)

B. *Equations of curves of response.*

The growth responses of the bucks and does respectively, which were used to construct the 5 weeks' curves of response already in use in this laboratory, have been used to determine the curves of response for 1, 2, 3, 4 and 5 weeks (Table II). For each curve, we included every rat alive at the end of each

Table II. *Mean increase in weight of groups of rats.*

Bucks					Does						
1 week	2 weeks	3 weeks	4 weeks	5 weeks	1 week	2 weeks	3 weeks	4 weeks	5 weeks	Dose	Log. of dose
−5·1	−8·1	−11·2	−12·9	−12·5	−2·5	−2·3	−1·5	−1·0	−3·3	0·25	−0·6021
0·5	4·8	7·3	11·5	13·5	−1·4	−0·8	5·0	10·1	13·3	1·0	0
−1·33	5·8	9·5	15·4	18·8	3·7	8·4	13·7	15·4	16·1	1·5	0·1761
3·45	10·5	16·8	24·2	30·6	3·8	10·5	14·7	19·4	22·6	2·5	0·3979
8·5	20·8	33·4	45·0	55·7	6·7	16·2	24·5	32·3	36·9	7·5	0·8751

week. We, therefore, had to modify our old 5 weeks' curves slightly to conform to this, for originally we had considered a rat to have survived the 5 weeks' test if it had lived 4 weeks + 4 days—*i.e.* more nearly 5 weeks than 4. Thus our 5 weeks' curves in this paper are not identical with the two published previously, although nearly so. The equations of the curves of response for the 5 periods are:

Duration of test, weeks	Bucks	Does
1	$y = -0{\cdot}33 + 9{\cdot}05 \log x$	$y = 0{\cdot}94 + 6{\cdot}64 \log x$
2	$y = 3{\cdot}5 + 19{\cdot}28 \log x$	$y = 4{\cdot}1 + 13{\cdot}45 \log x$
3	$y = 6{\cdot}04 + 29{\cdot}62 \log x$	$y = 8{\cdot}24 + 17{\cdot}93 \log x$
4	$y = 10{\cdot}2 + 38{\cdot}07 \log x$	$y = 11{\cdot}43 + 22{\cdot}48 \log x$
5	$y = 13{\cdot}36 + 45{\cdot}81 \log x$	$y = 12{\cdot}55 + 27{\cdot}0 \log x$

y = the mean increase in weight (g.) of the group of rats.
x = the dose (mg.) of cod-liver oil given daily.

They are drawn in Fig. 1 with the logs. of the doses instead of the doses as abscissae. It is obvious that the curves are not perfect, partly from the fact that the actual determinations do not lie exactly on the curves (though so nearly so that it is justifiable to draw the best straight line between them), and partly from the fact that, in each set, one curve is slightly out of place.

C. *The relation of the duration of the test period to the degree of accuracy obtainable by the test.*

The degree of accuracy of the test obtained in any given length of test period is determined partly by the standard deviation of the mean increase in weight for that period, and partly by the slope of the curve of response for that

period. A calculation involving these two factors has been made and summarised in Table III. It is based on an imaginary mean increase in weight of 5 g. in 1, 2, 3, 4 or 5 weeks. The following calculations for each period were made: (1) the abscissa of the appropriate curve of response corresponding to a mean increase in weight of 5 g., (2) the probable error of the mean increase in weight of a group of 10 rats, (3) the abscissae corresponding to the mean increase ± the probable error of the mean increase when $n = 10$, and (4) the percentage range of value of the abscissa (dose of vitamin A). From the last column of Table III it is evident that the range (*i.e.* the inaccuracy of the test) falls rapidly as the length of the test is extended from 1 to 2 weeks, less rapidly from 2 to 3 weeks and only slightly from 3 to 4 and from 4 to 5 weeks. Indeed the very small increase in accuracy gained by prolonging the test beyond 3 weeks does not, for most purposes, justify the extra time and labour involved.

Table III. *Accuracy of test related to duration of test period.*

Duration of test, weeks	Mean increase in weight (g.) say:	Abscissa of corresponding curve of response	Probable error ($\frac{2}{3}\epsilon$) of mean increase in weight when $n=10$	Range of abscissae corresponding to mean increase in weight $\pm\frac{2}{3}\epsilon$	% range of value
Bucks:					
1	5	3·8815	±1·44	5·5989	144·2
				2·6902	69·3
2	5	1·1962	±2·05	1·5280	127·7
				0·92534	77·3
3	5	0·92236	±2·50	1·1202	121·4*
				0·75947	82·3*
4	5	0·73014	±2·90	0·87015	119·2
				0·61277	83·9
5	5	0·65690	±3·22	0·77132	117·4
				0·55873	85·0
Does:					
1	5	4·0870	±1·19	6·1761	151·1
				2·7053	66·2
2	5	1·1665	±1·70	1·5607	133·8
				0·87198	74·8
3	5	0·65962	±2·06	0·85941	130·3
				0·50629	76·8
4	5	0·51761	±2·35	0·65841	127·2
				0·40690	78·6
5	5	0·52396	±2·52	0·64863	124·1*
				0·42228	80·6*

* These figures are probably not quite as accurate as the rest, as the two curves of response which were used for their calculation are slightly out of place (see Fig. 1).

Table IV. *Logarithmic variance of increases in weight at 1–5 weeks.*

	Duration of test, weeks	Standard deviation	Slope of curve of response	σ/slope = logarithmic variance
Bucks:	1	6·82	9·05	0·75
	2	9·68	19·28	0·50
	3	11·86	29·62*	0·40
	4	13·70	38·07	0·36
	5	15·32	45·81	0·33
Does:	1	5·63	6·64	0·85
	2	8·05	13·45	0·60
	3	9·74	17·93	0·54
	4	11·11	22·48	0·49
	5	11·93	27·0*	0·44

* These figures are slightly high (see Fig. 1). The corresponding values for the logarithmic variance are therefore slightly lower than they should be.

D. *The influence of the duration of the test on the logarithmic variance.*

The logarithmic variance of the increase in weight of the bucks and does in 1, 2, 3, 4 and 5 weeks has been estimated from the formula, $\lambda = \sigma$/slope of curve of response. The result is seen in Table IV. It demonstrates afresh that the degree of accuracy of the test is only slightly increased by prolonging the test period beyond 3 weeks.

SUMMARY.

A calculation has been made showing that the increase in accuracy obtainable by the prolongation of a vitamin A test (growth response after cessation of growth on a diet deficient in vitamin A) beyond a period of 3 weeks is too slight to justify generally the extra expenditure of time and labour which would be involved.

The probable error of an estimation made with 10 male rats is 44, 28, 21, 19, 17 % above, or 31, 23, 18, 16, 15 % below the true value at 1, 2, 3, 4 and 5 weeks respectively. Similarly the result obtained with 10 does is 51, 34, 30, 27, 24 % above, or 34, 25, 23, 21, 19 % below the true value at 1, 2, 3, 4 and 5 weeks respectively.

The calculation has been based on ample experimental evidence (2411 rats distributed over 201 tests) and, therefore, estimates the error from all sources. It has been worked out on accepted principles of statistical method.

REFERENCES.

Coward (1932). *Biochem. J.* **26**, 691.
—— Key, Dyer and Morgan (1930). *Biochem. J.* **24**, 1952.
—— —— —— —— (1931). *Biochem. J.* **25**, 551.
Norris and Church (1932). *J. Nutrition*, **5**, 495.
Sherman and Batchelder (1931). *J. Biol. Chem.* **91**, 505.

LXVI. THE DEGREE OF ACCURACY OBTAINABLE BY THE LINE TEST IN ESTIMATIONS OF VITAMIN D.

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(*Received February 23rd, 1933.*)

Bills *et al.* [1931] have estimated that, in the line test as carried out in their laboratory, the probable deviation from the mean for a large colony of rats, given by any group of 20 rats from the colony, is about 10 %. They determined this figure practically, by grouping, shuffling and regrouping results from 200 rats all given the same dose of irradiated ergosterol in tests carried out during 3 months of the spring of 1929. The figure they obtained (10 % for 20 rats), therefore, gave the limits (100 $\pm$ 10 %) within which a result might have been expected to lie once out of every 2 times of making an estimation during that particular spring when any 20 rats of that colony were used for the test.

The estimate of the probable error included the variation that may exist between different litters (inter-litter variation) as well as the variation between the members of any one litter (intra-litter variation). If Bills *et al.* had calculated the standard deviation from pairs of animals, both members of a pair belonging to the same litter, their estimate of the probable error might perhaps have been smaller. If, however, 10 % be accepted as the probable error of the mean degree of healing given by 20 rats, $10 \times \sqrt{2}$, which is about 14 %, may be accepted as the probable error of the estimation of one substance in terms of the other (*e.g.* an unknown oil in terms of the standard) made by the use of 20 pairs of rats, one rat of each pair being given the unknown, and the other rat of each pair being given the standard.

Bourdillon *et al.* [1931] found the probable error of the ratio of the potencies of 2 substances determined by the radiographic method as used in a curative test on 20 pairs of rats to be 8·2 %.

Bourdillon and Bruce [1932] used the radiographic method to estimate the degree of protection from rickets in rats [Hume, Pickersgill and Gaffikin, 1932] which had been given doses of vitamin D throughout the whole period of the experiment. By using 20 pairs of rats for a comparison between 2 doses of vitamin D, they obtained a probable error of the ratio of the potencies of the 2 doses equal to 12–15 % when the doses ranged from 0·05 to 0·2 unit. For lower or higher doses, the probable error was much greater. Similarly, the "ash content of the bones" method has a probable error of 13–16 % when the doses given range from 0·03 to 0·64 unit.

Morgan [1932] made large scale drawings of the cut surfaces of the bones of rats used in the line test by means of a lens and camera lucida. He then measured the areas of new calcification with a planimeter. He claimed a probable error of 5·4 % when 20 pairs of rats were used in a comparison.

The line test as carried out in our laboratories appears to have a probable error of 12 % when the doses of vitamin D given range from 0·2 to 1·5 unit. A somewhat greater degree of accuracy is obtainable by pairing rats as nearly as possible of equal initial weights.

Method of the test.

The technique of the line test has been fully described in previous papers from this laboratory [Dyer, 1931; Key and Morgan, 1932]. It may be very briefly summarised. Rats of 50–60 g. weight are fed on the Steenbock rachitogenic (2965) diet for $3\frac{1}{2}$ weeks. For a further period of 10 days one or two rats of each litter are given no addition to the diet, half of the rest of the rats of the litter are given equal doses of the test substance daily for 10 days and the other half are given equal doses of the international standard for vitamin D. The degree of healing of each rat is assessed by reference to a series of selected bones showing graded degrees of healing (assessed as 0–6 degrees); the results from all the rats given each dose are averaged and the relative vitamin D potencies of the 2 doses are compared by means of a curve drawn up in this laboratory relating degree of healing to dose of vitamin D given. The potency of the unknown is then expressed in international units, *e.g.* 5 rats given 5 mg. cod-liver oil showed degrees of healing of 4·5, 3, 3, 3·5, 4, respectively; 5 rats given 0·5 unit vitamin D showed degrees of healing of 3·5, 3, 2, 2·5, 3, respectively.

$$\frac{\text{The average healing from 5 mg. cod-liver oil}}{\text{The average healing from 0·5 unit D}} = \frac{3{\cdot}6}{2{\cdot}8}.$$

By substitution from the curve drawn up in this laboratory [Key and Morgan, 1932] relating degree of healing to dose of vitamin D given, the ratio $\frac{3{\cdot}6}{2{\cdot}8}$ for degree of healing corresponds to the ratio $\frac{0{\cdot}33}{0{\cdot}175} = 1{\cdot}9$ for dose of vitamin D given. Therefore 5 mg. cod-liver oil contain $1{\cdot}9 \times 0{\cdot}5 = 0{\cdot}95$ unit vitamin D, and the oil contains 190 units vitamin D per g.

All the litters from which information has been gathered for this paper showed a severe initial degree of rickets; only the lowest of the 3 curves of Key and Morgan's paper has, therefore, been used for calculations.

I. Accuracy of the test.

(*a*) *The mean variance of rats given the same dose of vitamin D.* Information concerning the accuracy of the line test has been obtained, in this laboratory, from the behaviour of pairs of rats (and sometimes 3 rats) of different litters, each pair (or 3) having received the same dose of vitamin D. The simplest way of determining the standard deviation of the degree of healing was, therefore, to apply the formula $\sigma^2 = \frac{\Sigma (x_1 - x_2)^2}{2N}$ where $x_1 - x_2$ is the difference between the degrees of healing of the 2 rats of each pair and N the number of pairs of rats. For collecting figures for the calculation of σ, in tests where we had 3 rats in a litter receiving the same dose, the first two as they occurred in the records were used, the third being ignored. In this way, no one rat occurs in the calculations twice and overweighting by any one rat is avoided. In the first calculation all the pairs were used regardless of sex and weight. The mean variance (σ^2) calculated from 626 rats (313 pairs) by means of this formula was 0·6135, whence the standard deviation (σ) is 0·78326. That is, the result from a third of the

rats used would be either more than the mean degree of healing *plus* 0·78 degree of healing, or less than the mean *minus* 0·78 degree of healing. The distribution of differences in degrees of healing of pairs of rats is shown in Fig. 1.

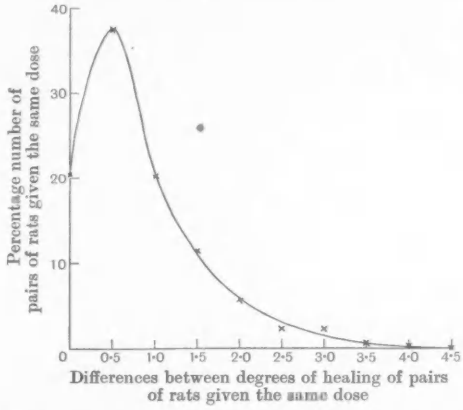

Fig. 1. Distribution of differences between degrees of healing of pairs of rats given the same dose.

Separate calculations of the standard deviation were made at this stage of all possible pairs of (*a*) bucks, (*b*) does, and (*c*) mixed pairs. The standard deviation of the does was distinctly less than that of the bucks (σ of bucks = 0·775 (114 pairs); σ of does = 0·667 (74 pairs); σ of mixed pairs = 0·823 (251 pairs)). When it was discovered later that the degree of healing was influenced by the initial weight of the rat, a fresh calculation of the standard deviations of the bucks and does was made using only those pairs of rats whose initial weights differed by not more than 2 g. This calculation gave:

σ (bucks) = 0·874 (from 34 pairs of rats).
σ (does) = 0·714 (from 27 pairs of rats).
σ (mixed pairs) = 0·812 (from 70 pairs of rats).

These figures are larger than those found when pairs were not selected according to initial weights, but the number of pairs of rats from which the calculations were made was very small; and, as tests can seldom be carried out entirely on bucks or on does, it would appear to be reasonable to take the original figure of 0·78 or approximately 0·8 as a general measure of the standard deviation of single animals from the mean in this test.

The value of the formula $\sigma^2 = \frac{\Sigma (x_1 - x_2)^2}{2N}$ in the determination of the variance of an estimation lies in the fact that the determination is made from pairs of animals, each pair belonging to one litter. Thus it is a measure of variation within litters only, which is the figure required for comparisons made always on litter-mate animals. A measure of the variation between animals of different litters is not required for this work. It was determined, however, from our tests for purely theoretical interest. For 26 bucks given 0·5 unit D, σ was found to be 0·90; for 26 does, σ = 0·97; for 78 bucks given 0·25 unit D, σ was found

to be 1·42; for 78 does, $\sigma = 1{\cdot}30$. The standard deviation of the response of animals belonging to different litters is evidently greater than that of animals belonging to the same litter.

(*b*) *Variations in the standard deviation for different degrees of healing.* When a given dose of vitamin D has brought about complete healing of a rachitic bone in a rat, it is possible that the value 6 (the highest in the scale) does not indicate the full potency of the dose. A smaller dose might have brought about 6 degrees of healing also. Thus the occurrence of one such rat in a group would lower the average degree of healing at this end of the scale, and also an estimation of the variance expressed in scale units at the upper end of the scale would be less than at the middle part. A similar consideration leads to the same conclusion for the lower part of the scale. This point has been investigated from the 313 pairs of rats used in the first estimation of the variance. The pairs of rats have been divided into three groups according as the lower degree of healing shown by a member of the pair lies between 0 and 2, 2·25 and 3·5 or 3·75 and 5·5 degrees of healing respectively, and the standard deviation of each group has been determined. The results are collected in Table I.

Table I.

No. of pairs of rats in group	Degree of healing of the less healed rat of each pair	Observed value of σ	Values of σ calculated from the relative numbers of pairs of bucks or does or mixed pairs according to	
			(*a*) the first calculation*	(*b*) the second calculation*
137	0–2	0·936	0·786	0·805
84	2·25–3·5	0·810	0·784	0·809
87	3·75–5·5	0·546	0·787	0·816

No pairs of rats in which both animals showed 0 degrees of healing have been included in these calculations.

* In I (*a*) of this paper.

That these values are not accounted for by differences in the relative numbers of pairs of bucks or does or of mixed pairs in each of the three groups is shown by a calculation of σ for each group from the standard deviation of bucks, does and mixed pairs, and the known numbers of each kind of pair in each group.

The value of σ, 0·546, found for the group showing the highest degrees of healing is much less than the average, 0·783, for the whole group, and may be accounted for by the argument of limits of healing given above. On the other hand the value, 0·936, for the group showing the lowest degrees of healing is much greater than the average value, which is contrary to the result expected from the argument on limits of healing. The significance of the difference in values of σ is considered in section (*d*).

(*c*) *The standard error* (ϵ) *of the mean.* In Table II is shown the method of determining the percentage error of an estimation of vitamin D obtained by the use of 20 rats all given the same dose. The average value for σ (0·783) has been used for the calculation as it is rightly applicable to the degree of healing selected for the calculation. The possibility of using all bucks or all does for a test is so small that no separate calculations have been made from the values found for bucks and does respectively. For the same reason we made no attempt to construct separate curves of response for bucks and does. The one in use in this laboratory was constructed from almost equal numbers of bucks and does on each dose, and later work described in this paper has shown that the responses of bucks and does to the same dose of vitamin D are equal.

Table II. *The accuracy of an estimation when 20 rats are all given the same dose.*

$$\epsilon = \frac{\sigma}{\sqrt{n}} = \frac{0{\cdot}783}{\sqrt{20}} = 0{\cdot}175.$$

Mean degree of healing	Abscissa of curve relating degree of healing to dose of vitamin D given	Range of degree of healing	Range of abscissae of curve	% range of equiv. doses
3·0 (say)	0·205	(*a*) $\pm\frac{2\epsilon}{3}$, 1 in 2 chance (probable error)		
		3·117	0·222	109
		2·883	0·187	91
		(*b*) $\pm\epsilon$, 2 in 3 chance		
		3·175	0·235	114
		2·825	0·177	86
		(*c*) $\pm 2\epsilon$, 21 in 22 chance		
		3·35	0·270	132
		2·65	0·152	74

(*d*) *The probable error for different degrees of healing.* In Table III are shown the probable errors of estimations for different degrees of healing, using the different values of σ found approximately for those degrees, 20 rats being used in each estimation. It may be concluded from the figures in the last column that a test giving a low average degree of healing is much less accurate than one giving about 2·0–4·5 degrees of healing. That the accuracy of the test at 4·5 degrees of healing is not greater than that at 3·0 degrees in spite of the standard deviation at 4·5 degrees being much less than at 3·0 degrees is accounted for by the shape of the curve relating degree of healing to dose of vitamin D given.

Table III. *The probable error for different degrees of healing calculated from the different values of σ for those degrees.*

Mean degree of healing	Value of σ for this degree of healing	Abscissa of curve relating degree of healing to dose of vitamin D given	Range of healing = mean $\pm\frac{2\epsilon}{3}$	Range of abscissae of curve	% range of equiv. doses
1·0	0·936	0·020	1·14	0·026	130
			0·86	0·014	70
3·0	0·810	0·205	3·12	0·225	110
			2·88	0·185	90
4·5	0·546	0·770	4·58	0·845	110
			4·42	0·700	91

(*e*) *The standard deviation of the difference between two results.* When each of two estimations may have a variance of σ^2, the variance of the difference between these two results is $2\sigma^2$ and, therefore, the standard deviation of the difference is $\sqrt{2}\sigma$. This is shown by the formula $\sigma^2 = \sigma_1^2 + \sigma_2^2 - 2r\sigma_1\sigma_2$ where σ_1 and σ_2 are the standard deviations of two variables and r the correlation between the variables.

In this work on vitamin D, σ_1 may in general be taken as equal to σ_2 and $r = 0$, hence the standard deviation of the differences between pairs of rats is $\sqrt{2}$ times the standard deviation of single rats from the same litter. Therefore, the average value of σ, 0·783, found in the first calculation, has been multiplied

by $\sqrt{2}$ to get the standard deviation, 1·104, of the difference between the two results. In Table IV is shown the accuracy obtainable in a comparison of two substances (one of which would probably be the standard and one an unknown substance) by the use of different numbers of pairs of rats, one rat of each pair being given a certain dose of standard, and the other a certain dose of the unknown.

We see in Table IV that, with 20 pairs of rats in a comparison, we obtain a result (in which we state the value of the unknown substance in terms of

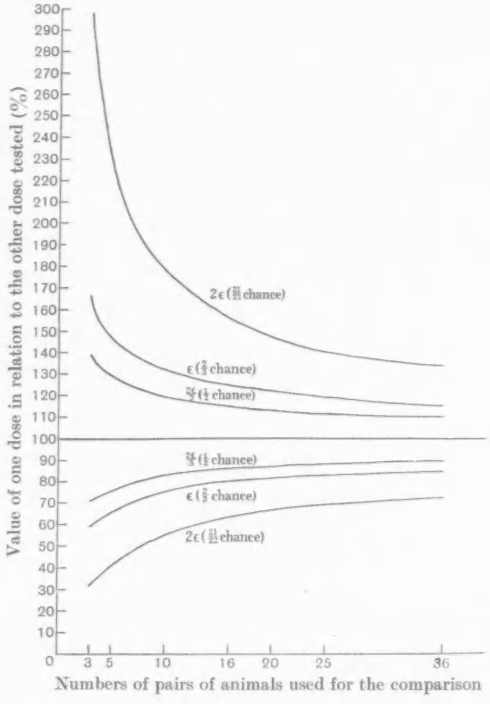

Fig. 2. Degree of accuracy obtainable in the ratio between the vitamin D content of two doses.

another substance on which a simultaneous test has been made) with a probable error of 13 %. This has been calculated from the value 0·783 for σ the average standard deviation we encounter in this test, *i.e.* the pairs of rats may consist entirely of bucks or of does, or they may be mixed pairs. A similar calculation using $\sigma = 0{\cdot}775$ (pairs of bucks only) makes a negligible difference to the probable error of 13 %. A calculation using $\sigma = 0{\cdot}667$ (pairs of does only, any weight) only reduces the probable error to 11 %. Thus 12 % may be considered a fair approximation to the probable error of the line test. Similarly the calculation of the range of values of the mean $\pm 2\epsilon$ shows that, with 20 pairs of rats

Table IV. *Degrees of accuracy obtainable in assessing one substance in terms of another on which a simultaneous test is made.*

Pairs of rats	Corresponding values of			Range of degrees of healing. Mean 3·0			Range of abscissae corresponding to the range of degrees of healing. Mean 0·205. Chances			% range of doses Chances		
	$\frac{2\epsilon}{3}$	ϵ	2ϵ	$\pm\frac{2\epsilon}{3}$	$\pm\epsilon$	$\pm 2\epsilon$	1/2	2/3	21/22	1/2	2/3	21/22
3	0·426	0·638	1·276	3·426	3·638	4·276	0·285	0·340	0·610	139	166	297
				2·574	2·362	1·724	0·145	0·120	0·065	71	59	32
5	0·330	0·494	0·988	3·330	3·494	3·988	0·265	0·302	0·470	129	147	229
				2·660	2·506	2·012	0·155	0·135	0·085	76	66	41
10	0·234	0·350	0·700	3·234	3·350	3·700	0·245	0·270	0·363	119	132	177
				2·766	2·650	2·300	0·170	0·153	0·113	83	75	55
16	0·184	0·276	0·552	3·184	3·276	3·552	0·235	0·255	0·320	115	124	156
				2·816	2·724	2·448	0·176	0·164	0·128	86	80	63
20	0·165	0·247	0·494	3·165	3·247	3·494	0·230	0·250	0·302	113	122	147
				2·835	2·753	2·506	0·178	0·167	0·138	87	81	67
25	0·148	0·221	0·442	3·148	3·221	3·442	0·228	0·245	0·288	111	119	140
				2·852	2·779	2·558	0·182	0·170	0·142	89	83	69
36	0·123	0·184	0·368	3·123	3·184	3·368	0·225	0·235	0·274	110	115	134
				2·877	2·816	2·632	0·185	0·174	0·150	90	85	73

$\epsilon = \frac{0{\cdot}78}{\sqrt{n}} \times \sqrt{2}$ = the standard error of the difference between two results.

in a comparison, a result will be obtained 21 out of 22 times within 33 % below or 47 % above the true value.

These probabilities have been shown graphically in Fig. 2.

II. Factors influencing the degree of healing produced by a given dose of vitamin D.

(*a*) *The weight of the rat at the beginning of the experiment,* i.e. *at the beginning of the 3–3½ weeks preparatory period.* Four separate calculations of the correlation between degree of healing and initial weight have been made, bucks and does being calculated separately lest the response of bucks to a given dose should be different from that of does. All the results available from bucks or does on doses of 0·25 or 0·5 unit D were used, irrespective of pairing. The calculations were based on the formula

$$\rho = \frac{Sxy}{\sqrt{S(x^2) \cdot S(y^2)}},$$

where x is the deviation from the mean initial weight, y is the deviation from the mean degree of healing.

The result is summarised in Table V.

Apparently there is a strong correlation between the degree of healing produced by a given dose of vitamin D and the weight of the rat at the time when it is first given the rachitogenic diet. It would, however, be unsafe to attach very great importance to an apparent correlation such as this in view of the lack of certainty that all other conditions of the experiment had not been changed, but correlation has been demonstrated in 2 groups of bucks and in 2 groups of does; it might be worth while, therefore, in arranging the rats of a litter for dosing, to pair those of weights as nearly equal as possible.

Table V. *Correlation between initial weight of rat and degree of healing brought about by a given dose of vitamin D.*

Group of rats given	No. of rats	Mean initial weight g.	σ of initial weight	Mean degree of healing	σ of degree of healing	Value of ρ	P probability that there is no correlation
0·5 unit D	54 ♂	60·6	7·21	4·4	0·88	0·3388	0·01
	41 ♀	58·8	4·13	3·99	1·17	0·2557	Very slightly $> 0·1$
0·25 unit D	128 ♂	60·4	6·28	2·85	1·44	0·2286	0·01
	133 ♀	57·0	4·76	2·2	1·27	0·2780	$< 0·01$

(*b*) *Correlation between sex and degree of healing.* To determine whether sex has any influence on the degree of healing produced in the line test, a comparison was made between pairs of rats which consisted of one buck and one doe from the same litter, which had received the same dose of any substance and (to eliminate the influence of weight) which did not differ by more than 2 g. in their initial weights.

We had 72 pairs fulfilling these conditions. The average degree of healing of the bucks was 2·96 and of the does 2·79. It was, therefore, concluded that sex had no influence on the degree of healing produced by a given dose of vitamin D. It was not considered necessary to work out the significance of the difference between the two results.

It is worthy of note that the first calculation we made of the correlation between sex and degree of healing did not allow for the differences in initial weights of the rats, and the degree of correlation was found to be significant. After making the calculation in (*a*) where difference in sex was eliminated, and difference in initial weight was shown to influence the degree of healing, it was obviously necessary to make a fresh calculation of the influence of sex, eliminating differences in initial weight. The influence of sex as described above was then found to be negligible.

(*c*) *Correlation between degree of healing and increase in weight during the 5 weeks' experimental period (3–3½ weeks preparatory + 10 days on dose of test substance).* To determine whether the increase in weight of a rat during the 5 weeks' experimental period had any influence on the degree of healing produced by a given dose of vitamin D, all available results were grouped according to (*a*) dose of vitamin D given, (*b*) sex, and (*c*) initial weight, three initial weights being chosen, *viz.* 54–56, 58–60 and 62–64 g. respectively. Thus 12 groups of animals were available for examination on this point, and in addition estimations were made of the bucks and does grouped together in each of the weight groups given 0·5 unit D. The correlation coefficient of each group was calculated. Results are summarised in Table VI.

It may be seen that in 3 of the groups there is a good indication of correlation between degree of healing and increase in weight during the test, but in the other 19 there is no indication of such a relationship. Moreover, 4 negative values of ρ would indicate, if they were big enough to be significant, that the greater the increase in weight, the less the degree of healing produced by a given dose, while the other positive results would indicate (if big enough) that the greater the increase, the greater would be the degree of healing. It must, therefore, be concluded that the increase in weight during the 5 weeks' experimental period has no influence on the degree of healing produced by a dose of vitamin D.

Table VI. *Correlation between degree of healing and increase in weight during 5 weeks' experimental period.*

No. of rats	Sex	Dose of vitamin D unit	Initial weight of rats g.	Average increase in weight g.	σ of increase in weight	Average degree of healing	σ of degree of healing*	ρ	P probability that there is no correlation†
1. 10	♂	0·5	54–56	22·9	4·17	3·9	0·76	0·205	>0·1
2. 13	♀	0·5	54–56	19·7	7·26	3·9	1·44	0·239	>0·1
1 and 2 estimated as 1 sample		0·5	54–56	21·1	6·20	3·9	1·17	0·229	>0·1
3. 18	♂	0·5	58–60	24·4	7·88	4·2	1·17	0·175	>0·1
4. 12	♀	0·5	58–60	23·0	6·22	3·5	1·07	−0·136	>0·1
3 and 4 estimated as 1 sample		0·5	58–60	23·9	7·16	3·9	1·17	0·117	>0·1
5. 9	♂	0·5	62–64	26·6	3·81	4·8	0·62	−0·073	>0·1
6. 9	♀	0·5	62–64	23·0	8·88	4·0	0·88	0·887	<0·01
5 and 6 estimated as 1 sample		0·5	62–64	24·7	6·90	4·4	0·83	0·685	<0·01
7. 32	♂	0·25	54–56	27·3	8·57	3·0	1·49	0·338	0·04
8. 52	♀	0·25	54–56	21·0	5·75	2·1	1·26	−0·062	>0·1
9. 34	♂	0·25	58–60	29·2	8·05	2·6	1·22	0·268	>0·1
10. 27	♀	0·25	58–60	28·0	7·28	1·8	1·22	0·457	0·02
11. 28	♂	0·25	62–64	34·0	11·14	2·6	1·36	−0·022	>0·1
12. 14	♀	0·25	62–64	25·9	5·83	2·6	1·39	0·181	>0·1

* This is a measure of the standard deviation of individuals in different litters as well as of some in the same litters. It is, therefore, not to be compared with the value 0·78 found for intra-litter variation.

† Fisher [1928; paragraph 34. Significance of an observed correlation, pp. 157, 174].

(*d*) *Abnormal growth of rats during the experimental period.* It has frequently been urged by workers on the line test that any rat which has lost even 2 g. in weight during the experimental period should be discarded since an abnormal result (too high a degree of healing) might be expected. In our experiments of the last 12 months we have only had 12 rats on a dose of standard which lost as much as 2 g. in weight during the 10 days' test period and 3 of these had also lost weight during the preparatory period. It may be seen in Table VII, in which the result from each of these rats is compared with the average of all

Table VII. *The degree of healing of rats with abnormal growth during the test period.*

Litter	Rat	Initial weight g.	Growth in test period g.	Degree of healing	Average healing of all rats on the same dose at same time of year
0·25 unit D:					
1125	6591 ♀	62	−2, −7 P.P.	6	3·84 October, 1931
1149	6699 ♀	56	−3	3·75	3·84 October, 1931
1204	7007 ♂	60	−6	6	2·72 November, 1931
	7008 ♂	69	−7	6	2·72 November, 1931
1320	7622 ♂	56	−7, −4 P.P.	0·5	1·74 January, 1932
1695	9551 ♀	52	−2	3	2·15 June, 1932
1784	24 ♀	54	−4	1	1·4 July, 1932
1775	9980 ♀	50	−2	1·5	1·4 July, 1932
1783	21 ♀	60	−3	0·5	1·4 July, 1932
0·5 unit D:					
1429	8182 ♂	56	−2	4·5	3·9 March, 1932
1428	8176 ♀	61	−2, −9 P.P.	6	3·9 March, 1932
1790	47 ♂	59	−2	1	3·1 July, 1932

results obtained at the same time, that a loss of even 4 g. has not brought about a greater degree of healing than was observed in other rats given the same dose, unless the loss in the test period was accompanied by a loss in the preparatory period (P.P.). Thus 2 rats which lost 6 and 7 g. respectively in the test period and 2 rats which lost 2 g. each in the test period and 7 and 9 g. respectively in the preparatory period gave abnormally high results. On the other hand one rat which lost 7 g. in the test period and also 4 g. in the preparatory period gave an abnormally low result. It would, therefore, appear advisable to discard rats which lose, say, 5 g. or more during the whole experimental period. A smaller loss does not seem to make any material difference. This work confirms earlier results published by Coward and Cambden [1929].

Further evidence on this point was sought in groups of rats in each of which (*a*) all rats had received equal doses of a vitamin-containing substance, and (*b*) one of the rats had lost weight while the other rat or rats had gained in weight during the test period. Only three such groups could be found in our records. Details of their behaviour are collected in Table VIII. In one of these groups a loss of 4 g. weight was accompanied by a greater degree of healing, but in the other two groups a loss of weight of 2 g. did not influence the result.

Table VIII. *The degree of healing of a rat which has lost weight during the test period compared with that of other rats in the same litter which gained in weight.*

Litter	Rat	Initial weight g.	Growth in test period g.	Degree of healing	Substance tested
1227	7123 ♀	59	+4	3	0·5 g. margarine
	7124 ♂	59	−4	4	
	7125 ♂	57	+4	2	
1297	7507 ♂	58	0	3	2·5 mg. cod-liver oil
	7508 ♂	55	−2	2·5	
	7509 ♂	55	+2	2	
1477	8433 ♂	68	−2	4·5	5 mg. cod-liver oil
	8434 ♀	66	+7	4·5	
	8435 ♂	62	+5	4·5	

Still further evidence was sought in the behaviour of rats which had been given no dose in each litter. Out of 258 such animals, 15 which had lost from 2 to 9 g. each during some part of the preparatory period showed no spontaneous healing at all, 6 which had lost from 1 to 7 g. each showed a few small foci of healing and 1 which had lost 6 g. had developed a very thin line of

Table IX. *Spontaneous healing in rats which had lost weight during the experiment.*

No. of rats	Loss of weight of each rat in preparatory period g.		Spontaneous healing if any
15	8, 9, 4, 3, 2, 4, 4, 2, 4, 2, 2, 4, 2, 4, 3		0
6	3, 3, 2, 7, 1, 2		Few small foci
1	6		1 (very thin line)
	Loss of weight during test period (no dose)		
4	2, 3, 6, 4		0
	Loss of weight during		
	(*a*) preparatory	(*b*) test period	
1	4	9	3·5 (thick line)
1	2	6	1·5 (thin line)

healing. Similarly 4 rats which had lost from 2 to 6 g. each during the test period showed no spontaneous healing, while 1 rat which had lost 4 and 9 g. in the two parts of the test respectively had 3·5 degrees of healing, and 1 rat which had lost 2 and 6 g. in the two parts of the test respectively, had developed a thin line, 1·5 degrees of healing (Table IX).

We therefore conclude that under the conditions of the test in this laboratory a loss of weight up to 4–5 g. does not affect the degree of healing of the bones of the experimental animals, though a greater loss than this may do so.

(*e*) *Apparent variation in sensitivity of the whole stock of animals.* The average degrees of healing of all the rats given 0·25 or 0·5 unit vitamin D each month have been calculated. It is obvious that some influence has changed the sensitivity of the rats to both the doses of vitamin D. It is evident also that the variation is not seasonal. When we found that the degree of healing was influenced by the initial weight of the rat, we calculated the average initial weight of each group of rats to see whether the changes in response could be attributed to changes in average initial weight. The results are summarised in Table X.

Table X. *Correlation between average response and the average initial weight of the rats each month.*

	Dose given = 0·25 unit D				Dose given = 0·5 unit D			
	No. of rats	Mean degree of healing	Mean initial weight g.	Mean initial age days	No. of rats	Mean degree of healing	Mean initial weight g.	Mean initial age days
1931:								
October	32	3·8	62·9	37·0	—	—	—	—
November	27	2·7	57·7	35·6	—	—	—	—
December	33	2·2	55·8	34·0	—	—	—	—
1932:								
January	25	1·7	58·0	34·7	—	—	—	—
February	13	1·4	59·4	34·9	12	4·0	58·5	35·8
March	—	—	—	—	59	3·9	60·4	35·3
April	24	3·4	60·2	36·9	18	5·2	65·6	36·3
May	42	2·9	58·5	32·2	—	—	—	—
June	50	2·2	58·3	34·1	—	—	—	—
July	15	1·4	55·9	32·2	6	3·1	58·8	32·0
August	—	—	—	—	7	4·2	59·9	34·5
September	—	—	—	—	21	4·1	62·2	33·1
October	—	—	—	—	22	2·7	57·6	31·4
November	—	—	—	—	45	2·8	58·4	36·0
December	—	—	—	—	17	2·6	58·6	31·7

It is evident that the variations in response from month to month follow, in part only, the variations in average initial weights of the rats. Obviously, therefore, the initial weight of the rat is not the only factor determining the degree of healing obtained by giving a particular dose of vitamin D.

In Table X have been included also the average ages of the rats at the beginning of the experiment. It is evident that the correlation between average initial age and degree of healing is not greater than that between average initial weight and degree of healing.

Sources of error.

The chief sources of error in an estimation of vitamin D by the line test lie in (A) the inherent variability in response of different animals to the same

treatment, (B) errors in judgment of estimation of healing, and (C) errors of technique in the giving of the daily doses of substance containing the vitamin.

The measure of the standard deviation determined experimentally and estimated in this paper is a measure of the total error from all these sources.

(A) *The inherent variability in the response of different animals to the same treatment.* As it is impossible to give all animals identical treatment, the measure of the inherent variability must be obtained as a residual error after all known sources of error have been estimated and subtracted from the total. Evidence has been presented in the paper that variation between litters is greater than variation between members of the same litter, but as comparisons are always (or should always be) made on "litter-mates," inter-litter variation need not be considered.

(B) *Errors in estimation of healing.* The degree of healing produced in 25 litters (189 rats) was determined by one observer at two different times with an interval between the determinations varying from 1 week to 4 months. All the bones of any one litter were photographed on one plate and estimations of healing were made not only by comparison with the series of partly healed bones, but also by comparisons with each other.

The following results were obtained:

No. of rats given same value in each estimation	...	...	101
No. of rats given values differing by 0·5 degree	...	...	81
No. of rats given values differing by 1·0 degree	...	...	7

$$\text{Average error} = \frac{81 \times 0{\cdot}5 + 7 \times 1{\cdot}0}{101 + 81 + 7} = 0{\cdot}25 \text{ degree of healing.}$$

(In a routine test where 3–4 litters are used for an estimation, comparisons are made not only between members of any one litter, but also between the rats of all the litters; hence the average error in any test would probably be less than the general average just found.)

The standard deviation of a single determination of the degree of healing was calculated from the formula $\sigma^2 = \frac{\epsilon (x_1 - x_2)^2}{2N}$ where $x_1 - x_2$ is the difference between successive values assigned to any one rat, and N is the number of rats. The value of σ was found to be 0·27.

The standard error of the determination when 20 rats are used is thus 0·060 which, multiplied by $\sqrt{2}$, gives 0·085 as the standard error of the difference between two determinations. This corresponds to an error of 5 % in dosage which, therefore, accounts for about 40 % of the total error of a determination in which 20 pairs of rats were used.

(C) *Errors of technique in daily dosing.* In giving a daily dose of a substance of high vitamin potency to each of a large number of animals, certain measures have to be taken to reduce the labours of manipulation; for example, the dose of any sample of cod-liver oil which is required to obtain a measurable result in this test is so small that it cannot be measured easily for each rat each day. The weighing of a dose of 5 mg. would not be done without a certain amount of error and would be so laborious that other methods of measuring the daily dose are resorted to by various workers. In this laboratory we dilute our oil or the standard preparation of vitamin D with olive oil to such a degree that 0·02 g. of the solution contains the amount required for the daily dose. The storing of these dilutions and frequency of making have already been described [Dyer, 1931; Key and Morgan, 1932]. Doses are given daily from small dropping tubes selected for delivery of 19·5–20·6 mg. olive oil.

(*a*) *Errors in making the dilution of oil.* We have generally diluted a cod-liver oil to the required strength with olive oil by dropping the required number of drops of each oil from the same pipette, and then mixed the two oils by stirring with a finely drawn out glass rod for 4–5 minutes. The larger the number of drops of either oil, the less would be the error due to difference in size of the drops. Where the number of drops is small, as in the dilution of the standard, the error would be larger. An estimation of the error in the size of drops was made from the figures gained by selecting the dropping tubes for dosing. 194 single drops from 63 different dropping tubes were weighed, the variance from the mean for each tube calculated, and the average variance determined from the formula $\sigma^2 = \frac{\Sigma d^2}{N-m}$ where d is the deviation from the mean of each determination, N is the number of observations (*i.e.* weighings) and m the number of means (*i.e.* dropping tubes).

The standard deviation was found to be 0·53 mg. per drop of 20 mg. Thus when only 1 drop of a solution is used (which seldom or never happens) there is only a 1/22 chance of the drop being more than $20 + 2 \times 0{\cdot}53$ mg. or less than $20 - 2 \times 0{\cdot}53$ mg. That is, the error would almost never be greater than 5 %. If 2 drops are used, as in the dilution of the standard, the 1/22 error would be $\pm 2 \times \frac{0{\cdot}53}{\sqrt{2}} = 0{\cdot}75$ mg. and the error would be about 3·7 %. The probable errors corresponding to these figures are 1·7 and 1·2 % respectively.

Similarly, when 25 drops are used (a convenient number for a cod-liver oil dilution for a vitamin D test), the 1/22 error would be only $2 \times \frac{0{\cdot}53}{\sqrt{25}} = 0{\cdot}21$ mg. or 1 % and the probable error 0·33 %.

(*b*) *Errors in dose due to variation in size of drop given to rat.* The standard deviation of the size of a drop of 20 mg. was found to be 0·53 mg. Thus, for a 10 days' test, the standard error was $\frac{0{\cdot}53}{\sqrt{10}} = 0{\cdot}17$ mg. and the probable error, 0·11 mg. The dropping tubes delivered not less than 19·5 mg. and not more than 20·6 mg. Thus, when one of the smaller ones was used, 1 out of 2 times the drop would be either less than 19·39 mg. or greater than 19·61 mg. It would be less than 19·39 mg. only once in four times. Similarly it would almost never (1 out of 22 times) be less than 19·16 mg. or greater than 19·84 mg. So also, when one of the largest tubes was used, the limits would be 20·26 and 20·94 mg. respectively. As, however, the same tube is used for any dose throughout a 10 days' test, the probable error of the 10 days' dose may be stated as about 0·5 %. There is a 1/22 chance of an error of 1·7 %.

The difference in size of the dropping tubes would, of course, affect the accuracy of a comparison between two substances. To estimate this source of inaccuracy in a comparison, the data relating to 59 different dropping tubes which have been used in these laboratories have been examined. The average sizes of the drops from each of the 59 tubes have themselves been averaged and the standard deviation from this mean estimated. This proved to be 0·35 mg. whence it follows theoretically that half of the tubes deliver an average drop of 19·76–20·24 mg., two-thirds deliver an average drop of 19·65–20·35 mg. and twenty-one twenty-seconds deliver 19·3–20·7 mg. (Actually, tubes delivering 19·3 or 20·7 mg. were not used.) Thus the use of different dropping tubes gives a probable error in the difference between two substances of 1·7 % ($\sqrt{2}$ times 1·2). It cannot actually give a greater error than $\frac{20{\cdot}6 - 19{\cdot}5}{19{\cdot}5} \times 100$ which is 5·1 %.

(*c*) *Variation in size of drop with change of temperature.* A single determination of the influence of temperature on the size of a drop of olive oil was made.

Three estimations each of 5 drops of olive oil at 12° were respectively 0·1073, 0·1085 and 0·1075 g., average = 0·1078 g. Three estimations of 5 drops (from the same tube) of olive oil at 23° were respectively 0·1056, 0·1082, 0·1046 g., average = 0·1061 g. Thus a difference of 11° makes a difference of only 1·6 %. This is the greatest difference in temperature likely to be encountered between giving the doses as soon as possible after bringing the oils into the warm animal room and giving them towards the end of the time taken in dosing.

(*d*) *Slope of pipette during delivery of drop into rat's mouth.* It is necessary to hold the pipette vertically when letting the drop of oil fall into the rat's mouth. Variations in the size of the drop resulting from variation in the slope of the tube were determined in this laboratory 2 years ago. It was found that holding the tube at an angle of 45° with the vertical (an extreme position) may increase the size of the drop as much as 12 %. This error is not applicable to the present estimation of the degree of accuracy of the test for all the data here used were collected after this source of error had been determined and corrected.

(*e*) *The rapidity with which successive drops are allowed to fall* from a tube is known to influence the size of the drop. As dilutions of oils are so made that the required dose is contained in 1 drop, only 1 drop has to be let fall from the tube for each rat, and a skilled worker releases the drop of oil at a fairly constant rate for successive rats. We did, however, make one attempt to measure the variation in weight of a drop due to such variations in the rate of dropping as might occur in the making up of a dilution of the standard. When the drops were allowed to fall "quickly" (about 20 drops in 8 seconds) from a particular pipette the average weight was 29·2 mg. and when allowed to fall "slowly" (about 1 drop per second), the average weight was 30·4 mg. Variation in speed of releasing drops could, therefore, make an error of 4·1 %. In preparing a dilution of the standard for feeding, the few drops of standard would be allowed to fall slowly, but the larger number of drops of olive oil would be allowed to fall quickly. Thus, in a 1 in 40 dilution, the standard might be about 4 % weaker than was intended, but successive dilutions would be of almost equal strength. It would unquestionably be preferable to make such high dilutions as this by weight.

Summary.

The degree of accuracy obtainable by means of the line test as carried out in this laboratory has been estimated from an examination of the results from 956 rats.

It has been determined that, by using 20 pairs of rats for a comparison of the vitamin D content of two substances, the result will be within 12 % of the true relation 1 out of 2 times. It will lie within 33 % below (or 47 % above) the true relation 21 out of 22 times. When 10 pairs are used, the corresponding figures are 17 % below (or 19 % above), 1 out of 2 times, and 45 % below (or 77 % above) 21 out of 22 times.

The degree of healing produced by a dose of vitamin D appears to be influenced by the initial weight of the rat. Thus for comparison of two substances rats may be paired according to initial weight. Does and bucks respond equally to equal doses of vitamin D; hence sex need not be considered in arranging pairs of rats for tests.

Variations in increase in weight during the 5 weeks of the test (including preparatory period) do not influence the degree of healing produced by a given dose of vitamin D. Loss of weight during either the preparatory or the test period produces spontaneous healing only if the loss is more than 5 g.

The apparent variation in sensitivity of the whole stock of rats observed during the last 12 months seems to follow partly, though not wholly, changes in the average initial weights of the rats from month to month.

The chief sources of error in the dilution of standard and substance being tested and in the method of giving doses to the rats have been explored and estimated.

REFERENCES.

Bills, Honeywell, Wirick and Nussmeier (1931). *J. Biol. Chem.* **90**, 619.
Bourdillon and Bruce (1932). *Biochem. J.* **26**, 506.
—— —— Fischmann and Webster (1931). *Med. Res. Coun. Lond. Sp. Rep. Ser. No.* 158. (H.M. Stationery Office, London.)
Coward and Cambden (1929). *Quart. J. Pharm. Pharmacol.* **2**, 44.
Dyer (1931). *Quart. J. Pharm. Pharmacol.* **4**, 503.
Fisher (1928). Statistical methods for research workers, 2nd ed. (Oliver and Boyd.)
Hume, Pickersgill and Gaffikin (1932). *Biochem. J.* **26**, 488.
Key and Morgan (1932). *Biochem. J.* **26**, 196.
Morgan (1932). *Biochem. J.* **26**, 1144.

LXVII. FURTHER STUDIES OF THE EFFECT OF THE MEDIUM ON THE PRODUCTION OF BACTERIAL GELATINASE.

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(*Received February 27th, 1933.*)

In earlier work [Haines, 1932] it was shown that a measure of the activity of the gelatinase produced by bacteria growing in a given medium could be obtained by following the change in viscosity of a mixture of gelatin and sterile centrifugate from the medium. This change in viscosity is large and with an active centrifugate may be of the order of 50 %. The equation to the curve obtained by plotting change in viscosity against time was however unknown, and a purely arbitrary comparison was made by selecting the time required for 25 % change in viscosity of gelatin-enzyme mixture with centrifugates from the different media employed. Such a method was sufficiently accurate for the purpose in hand, but it has since been found possible to calculate a constant k characteristic of the activity of the centrifugate, and hence to determine with more precision the effect of factors which may cause only small variations in gelatinase production, *e.g.* the effect of varying the concentration of the salts of calcium and magnesium present.

It was at first hoped that the rate of change of viscosity might be a linear function of time over the early stages of the reaction. In order to test this, smaller viscometers than those used previously were made, giving readings of the order of 20–30 seconds. The experimental technique was standardised throughout and, provided the same procedure was always followed, good agreement between duplicate experiments could be obtained. A batch of phenol-gelatin, made up as described in the previous paper, was tubed in approximately 10 cc. lots so that each sample should have been melted the same number of times for the same period. The tubes were stored in the dark to prevent oxidation of the phenol, and enough for the work in hand only was made up, as irregular results were given with phenol-gelatin which had been kept for several weeks. When carrying out a determination, a tube of phenol-gelatin was melted by immersing for 5 minutes in a beaker of water at 37° in the incubator. The centrifugate from the culture was kept in this incubator for 30 minutes before use. 2·5 cc. of the culture were added to 5 cc. phenol-gelatin, using pipettes stored at 37°, the mixture shaken thoroughly and 5 cc. measured into the viscometer previously suspended in the thermostat at 37°. Exactly 4 minutes were allowed for the mixture to regain the temperature of the bath. If a longer time is allowed, the early stages of the reaction are missed and with an active centrifugate the relation outlined below may cease to hold after about 10 minutes. If, on the other hand, readings are begun while the temperature of the mixture is below 37° the values obtained will be spuriously high. By suspending a thermometer in the viscometer it was found that the mixture regained the temperature of the bath in about 3 minutes, but in order to avoid error a period of 4 minutes was

adopted. Readings were then begun by starting two stop-watches simultaneously at the beginning of the first observation. The time of fall of the mixture was read to the nearest 1/10 second with one watch, and the mean time of making the observation to the nearest second with the other watch. A suitable number of readings were then taken consecutively during the first 15 minutes or so of the reaction, usually at intervals of about 1 minute. An example of the results obtained when percentage change of viscosity is plotted against time is shown in Fig. 1. It will be seen that the relation between change of viscosity and

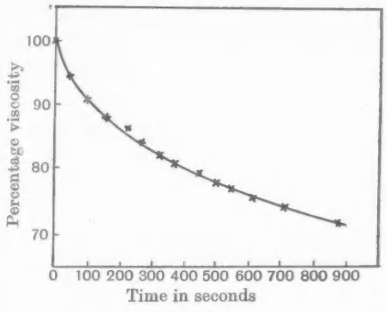

Fig. 1.

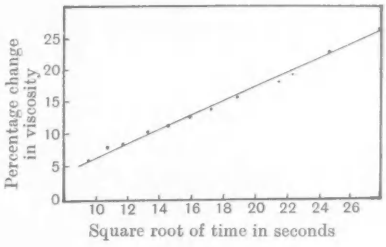

Fig. 2.

time is not linear. But if the change of viscosity be plotted against square root of time, a straight line results, so long as the observations are confined to the first 15 minutes or so of the experiment. That is to say, the action of crude gelatinase preparations over the early period follows Schütz's law [1885]. The values obtained for k assuming this relation and their standard deviation from

Table I. *Example of the values obtained for* k *assuming a linear relation between change of viscosity and square root of time.*

$$k = \frac{V_2 - V_1}{\sqrt{t_2} - \sqrt{t_1}}.$$

Time secs.	Viscometer reading secs.	Viscosity %	Change of viscosity %	$\sqrt{t}$	k calculated	Deviation
16	31·0	100·0	—	—	—	—
83	29·8	96·2	3·8	9·1	—	—
149	28·6	92·3	7·7	12·1	1·26	0·01
218	27·6	89·1	10·9	14·8	1·25	0·02
292	26·4	85·2	14·8	17·1	1·38	0·11
437	25·0	80·7	19·3	20·9	1·28	0·01
500	24·5	79·2	20·8	22·4	1·32	0·05
611	23·4	75·5	24·5	24·7	1·28	0·01
670	23·2	74·8	25·2	25·9	1·22	0·05
734	23·0	74·3	25·7	27·1	1·23	0·04
797	22·5	72·6	27·4	28·2	1·23	0·04
912	21·8	70·3	29·7	30·2	1·23	0·04
1008	21·4	69·0	31·0	31·7	1·20	0·07
1066	21·2	68·4	31·6	32·7	1·18	0·09
1211	20·4	65·8	34·2	34·9	1·12	0·15

Mean up to line = 1·27. Standard deviation = 0·015 = 1·1 %.

All readings below the line discarded as the linear relation ceases to hold.
Pseudomonas (*B. fluorescens*) organism, 3 days in broth at 20°.

the mean are given in Table I. The graph of square root of time plotted against change of viscosity is shown in Fig. 2. The agreement is sufficiently good for this relation to be used as a means of finding k for centrifugates from various media and thus obtaining a quantitative comparison.

Results with broth cultures.

Some figures obtained by this technique for organisms growing in nutrient broth p_H 7·5 are given in Table II. Representatives of most of the groups of aerobic bacteria which readily produce gelatinase have been studied. It will be seen that of the organisms tested *Pseudomonas* and *B. pyocyaneus* produce

Table II. *Gelatinase activity of broth cultures. Organisms grown in nutrient broth* p_H *7·5 at 20° except where otherwise stated.*

Organism	Source	Age of culture	Gelatinase activity of centrifugate k
Achromobacter Strain 5	"Slimy" meat	10 days	0·28
,, ,, 3	,,	8 ,,	0·48
B. subtilis	Lister Institute (37°)	6 ,,	0·46
,,	,, (20°)	7 ,,	0·45
B. mesentericus	Pathological Laboratory, Cambridge	3 ,,	0·82
,,	,,	12 ,,	1·01
B. proteus	Dung	12 ,,	1·04
Pseudomonas (*B. fluorescens*)	,,	3 ,,	1·20
,,	,,	12 ,,	1·46
,,	,,	40 ,,	1·11
Pseudomonas aeruginosa (*B. pyocyaneus*)	Lister Institute	7 ,,	1·15
,,	,,	36 ,,	1·48
,,	,,	9 weeks	1·04
Staphylococcus aureus	,, (37°)	6 days	0·07

the most active gelatinase, with *B. proteus* and *B. mesentericus* following closely. While this last organism produces an active gelatinase, the related *B. subtilis* is comparatively feeble in this respect. Representatives of the *Achromobacter* group which occur in large numbers on lean meat [Haines, 1933] are about as active as or rather less active than *B. subtilis*, whilst the least active organism is the *Staphylococcus* used.

The effect of p_H.

In the previous communication [Haines, 1932] it was shown that the optimum p_H for gelatinase action is at 8. This finding has been confirmed and extended by the use of the present technique. The value of k for a centrifugate from a 6-day culture of *B. proteus* in Merrill and Clark's medium was found when the centrifugate was incubated with gelatin made up in suitable buffers at p_H values ranging from 4 to 10. The results are shown graphically in Fig. 3 in which the value of k as a percentage of its maximum value is plotted against p_H.

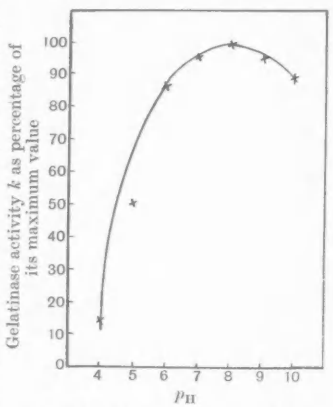

Fig. 3. Effect of p_H on gelatinase activity.

Results with synthetic media.

In the previous work the effect of salts of calcium and magnesium on the production of gelatinase was investigated, using a variety of media and different sources of nitrogen, chiefly with a strain of *B. proteus*. With minor variations the results in different media were essentially similar, namely, that salts of magnesium stimulate growth but not gelatinase production and may in some

Table III. *Gelatinase activity of centrifugates from Merrill and Clark's medium.*

Organism	Added salts	Growth*	Number of sub-culture	Age in days	k
Pseudomonas (*B. fluorescens*)	Ca + Mg	*M*/80	1	3	0·34
,,	Mg	*M*/80	1	3	0·00
,,	Ca + Mg	*M*/80	2	5	1·28
,,	Mg	*M*/80	2	5	0·00
,,	Ca + Mg	> *M*/80	4	12	1·36
,,	Mg	> *M*/80	4	12	0·00
B. mesentericus	Ca + Mg	*M*/80	1	3	0·50
,,	Mg	*M*/80	1	3	0·31
,,	Ca + Mg	> *M*/80	2	6	1·00
,,	Ca + Mg	> *M*/80	4	12	1·14
,,	Mg	> *M*/80	4	12	0·00
,,	Ca + Mg	> *M*/80	6	14	1·05
,,	Mg	> *M*/80	6	14	0·00
,,	Ca + Mg	> *M*/80	9	9	0·85
B. proteus	Ca + Mg	*M*/80	1	6	0·80
,,	Mg	*M*/80	1	6	0·00
,,	Ca	*M*/1000	1	6	0·50
,,	None	*M*/1000	1	6	0·00
,,	Ca + Mg	> *M*/80	3	14	1·18
,,	Mg	> *M*/80	3	14	0·08
,,	Ca	*M*/800	3	14	0·52
,,	None	*M*/800	3	14	0·15
Achromobacter (Strain 3)	Ca + Mg	> *M*/80	1	14	0·64
,, ,,	Mg	> *M*/80	1	14	0·00
,, ,,	Ca	*M*/800	1	14	0·45
,, ,,	None	*M*/800	1	14	0·00
,, ,,	Ca + Mg	> *M*/80	2	14	0·52
,, ,,	Ca	*M*/1000	2	14	0·02
Pseudomonas aeruginosa (*B. pyocyaneus*)	Ca + Mg	> *M*/80	1	9	1·52
,,	Ca	*M*/320	1	9	1·44
,,	Mg	> *M*/80	1	9	0·40
,,	None	*M*/320	1	9	0·53
,,	Ca + Mg	*M*/80	1	6	1·00
,,	Ca	*M*/640	1	6	0·90
,,	Ca + Mg	*M*/80	2	3	0·95
,,	Ca	*M*/1000	2	3	0·24
,,	Ca + Mg	> *M*/80	3	7	1·43
,,	Ca + Mg	> *M*/80	4	4	1·34
,,	Ca	*M*/640	4	4	1·25
,,	Mg	> *M*/80	4	4	0·00
,,	None	*M*/800	4	4	0·32

* The convention used to indicate amount of growth in Tables III and IV is the same as in the preceding paper [1932].

instances partly inhibit the latter, while salts of calcium without affecting growth greatly stimulate the production of gelatinase. In the present investigation, therefore, attention has been confined to one medium and a variety of organisms have been used. The medium chosen was the simplest one, that of Merrill and Clark [1928], using ammonium chloride as the source of nitrogen. Carefully purified substances were used and the medium made up as described in the earlier work except that calcium chloride crystallised *in vacuo* was

preferred to precipitated calcium carbonate in order to avoid the possibility of error introduced by withdrawing small quantities of a suspension of the carbonate. Values of k have been found for centrifugates of various ages from cultures of five organisms, and a selection of the results is given in Table III. It will be seen that the findings of the earlier work are confirmed with all five organisms. In every case good growth and the best production of gelatinase take place in the presence of salts of both calcium and magnesium. In the presence of magnesium salts only growth is as good as with both salts, but the production of gelatinase is poor and in many cases nil. In the presence of calcium salts alone, on the other hand, although growth is poor, appreciable gelatinase formation can be demonstrated. In certain instances the inhibitory effect of magnesium salts was again observed, *e.g.* in the third sub-culture with *B. proteus* and in most of the tests with *B. pyocyaneus* (Table III) the value of k in the presence of magnesium salts (good growth occurring) was less than that obtained in the absence of both salts (poor growth) and markedly less than k in the presence of calcium salts only (poor growth). With the latter organism, it will be seen that the value of k for the medium containing calcium salts only was higher compared with k for the medium containing both calcium and magnesium salts than with the other organisms used. The reason for this is obscure but may possibly be associated with the marked change in p_H which sometimes occurs in cultures of *B. pyocyaneus* (from 7·5 to 5 or 4, despite the buffering power of the phosphates present). In media containing calcium salts only growth was poor and the p_H remained steady. It might be thought at first sight that herein lay a reason for the non-production of gelatinase in media containing salts of magnesium only, but that this is not the case is shown by the fact that a value of 0·87 was obtained for k from a medium containing both salts even when the p_H had fallen to 4 approximately. Merrill and Clark [1928] also noted quite good gelatinase production with *B. proteus* even though the medium was finally quite acid.

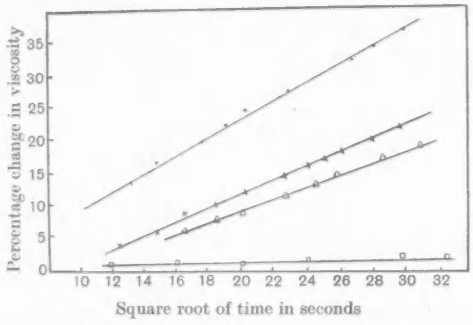

Fig. 4. Effect of varying the concentration of Ca and Mg salts.

• $CaCl_2 = MgSO_4 = 0{\cdot}002\,\%$; × $CaCl_2 = MgSO_4 = 0{\cdot}01\,\%$; △ $CaCl_2 = MgSO_4 = 0{\cdot}02\,\%$; □ No $CaCl_2$, $MgSO_4 = 0{\cdot}002\,\%$.

The effect of varying the concentration of the salts of calcium and magnesium.

Preliminary experiments showed that a concentration of either calcium or magnesium salts above about 0·04 % could not be used, owing to precipitation, presumably of phosphates, in the medium. Tests were carried out, therefore, at

concentrations ranging from 0·04 to 0·0002 % approximately, varying both salts together, and varying each singly with the other constant. The concentrations were obtained by dilution from stock 1·0 and 0·1 % solutions. The washed organism was inoculated into flasks of the medium and determinations of gelatinase made as described previously, results from at least two subcultures being obtained. The value of k was found graphically by taking the best straight line through all the points during the period in which the linear relation held. An example of the results is shown in Fig. 4 and the values of k are grouped together in Table IV. *Pseudomonas* (*B. fluorescens*) was the organism used.

Table IV. *The effect of varying the concentration of salts of calcium and magnesium in Merrill and Clark's medium. Reckoned in percentages of crystalline calcium chloride and magnesium sulphate.* Pseudomonas (B. fluorescens) *organism.*

Ca %	Mg %	Number of subculture	Age in days	Growth	k
0·04	0·002	1	5	*M*/80	0·86
0·02	0·002	1	5	*M*/80	0·91
0·01	0·002	1	5	*M*/80	0·52
0·002	0·002	1	5	*M*/80	0·42
0·0002	0·002	1	5	*M*/800	0·00
0·04	0·002	3	7	*M*/80	1·19
0·02	0·002	3	7	*M*/80	1·33
0·01	0·002	3	7	*M*/80	1·24
0·002	0·002	3	7	*M*/80	1·25
0·0002	0·002	3	7	*M*/800	0·00
0·002	0·04	1	6	*M*/80	0·90
0·002	0·02	1	6	*M*/80	0·86
0·002	0·01	1	6	*M*/80	0·81
0·002	0·002	1	6	*M*/80	0·93
0·002	0 0002	1	6	*M*/800	0·00
0·002	0·04	2	7	*M*/80	1·24
0·002	0·02	2	7	*M*/80	1·18
0·002	0·01	2	7	*M*/80	1·20
0·002	0·002	2	7	*M*/80	1·27
0·002	0·0002	2	7	*M*/800	0·00
0·02	0·02	1	7	*M*/80	0·90
0·01	0·01	1	7	*M*/80	1·05
0·002	0·002	1	7	*M*/80	1·34
0·0002	0·0002	1	7	*M*/800	0·00
0·02	0·02	2	7	*M*/80	0·99
0·01	0·01	2	7	*M*/80	0·91
0·002	0·002	2	7	*M*/80	1·53
0·0002	0·0002	2	7	*M*/8000	0·00
0	0·002	1	7	*M*/80	0·00
0	0·002	3	10	*M*/80	0·00

It will be seen that the production of gelatinase ranges from nil to its optimum value according to the salt content of the medium, growth being as good in the medium in which no gelatinase is produced (no calcium chloride and magnesium sulphate = 0·002 %) as in that in which best formation of gelatinase occurs (calcium chloride = magnesium sulphate = 0·002 %). The absolute values of k vary from subculture to subculture as is only to be expected, depending on the time of incubation, amount of growth, *etc.* The relative values

of k seem, as far as can be judged from these few experiments, to be similar in each set. Within the range 0·04–0·002 % varying the concentration of either calcium or magnesium salt, keeping the other constant at 0·002 %, does not seem very greatly to affect the gelatinase. When the concentration of calcium chloride falls below 0·002 %, however, gelatinase formation is small or zero. With magnesium sulphate at 0·002 % and no calcium chloride growth is excellent but gelatinase production nil. Varying the concentration of both salts together, the optimum seems to lie not in the most concentrated solutions but at the greatest dilution consistent with good growth.

The effect of continued subculture on the production of gelatinase.

Although the organisms were centrifuged and washed three times before inoculation into the synthetic medium, it was felt desirable to test gelatinase production in a series of subcultures in order to eliminate the possibility either of having carried over minute amounts of substances from the original culture or of some of the original inoculum which had remained dormant still persisting in the subcultures. *B. pyocyaneus* has been kept in broth culture for about 8 months, subculturing at intervals of about 14 days. *B. mesentericus* was taken through 9 subcultures over a period of 4 months in the synthetic medium, and *B. pyocyaneus* has been under observation during 10 subcultures in the synthetic medium over a period of about 6 weeks. The latter organism was subcultured rather more rapidly, every 3–4 days, to prevent interference by the large amounts of acid which are evident in older cultures. Gelatinase tests were carried out at intervals and the results are summarised in Table V.

Table V. *Effect of subculturing on the production of gelatinase.*

Number of subculture	Total age of culture in days	Age of subculture tested in days	k
B. pyocyaneus (*Pseudomonas aeruginosa*) in nutrient broth p_H 7·4:			
1	7	7	1·15
2	30	18	0·97
8	106	36	1·48
11	138	13	1·16
14	165	8	1·20
15	187	12	1·18
B. pyocyaneus (*Pseudomonas aeruginosa*) in Clark's medium with Ca and Mg salts:			
1	6	6	1·00
2	9	3	0·95
3	16	7	1·43
4	20	4	1·34
5	24	4	1·18
6	27	3	1·10
7	33	5	1·23
10	55	5	1·20
B. mesentericus in Clark's medium with Ca and Mg salts:			
1	3	3	0·71
2	41	6	1·00
4	54	12	1·14
6	82	14	1·05
9	102	9	0·88

It will be seen that no significant diminution in activity of the gelatinase present in the centrifugates from the cultures occurred over the periods studied.

Discussion.

A survey of the results of this and the preceding papers indicates that without exception all the organisms employed in all the media tested are greatly influenced in their capacity for producing gelatinase by the presence of salts of calcium and magnesium. The effect of these salts is, moreover, independent of their growth-stimulating properties. While it is true, as Wilson [1930] pointed out, that a certain amount of growth must take place before production of enzyme can be demonstrated, the position taken up by her that growth is the predominating factor and that these salts affect gelatinase formation only in so far as they affect growth does not seem to be tenable. The clearest proof of this is the fact that salts of magnesium alone, added to the synthetic medium, stimulate growth to its maximum, but so far from stimulating gelatinase production in some cases actually inhibit it. These effects, too, are not confined to one organism or one medium but appear to be of a fundamental nature, common to all, and to persist after repeated subculture in the medium. In a very simple medium, with ammonium chloride as the source of nitrogen and a suitable salt content, gelatinase is produced as readily as in the complex amino-acid mixture present in ordinary meat broth. The nature of the action of these salts is obscure. Apparently it is not a question of the activation of an enzyme produced in inactive form in the medium, since addition of salts of calcium to centrifugates from inactive cultures had no effect. It has been suggested that these salts may exert a permeability effect [Stephenson, 1930]. The experiment was therefore tried of adding calcium chloride to a thick suspension of the washed organisms in phosphate buffer, in the hope that if this salt were altering the permeability of the cell wall any pre-formed enzyme present might pass out and be demonstrable in the centrifugate. After 3 days' incubation the result was negative. While affording no evidence for that view this experiment does not prove the contrary, since possibly salts of calcium alter the permeability of the cell wall only in the young actively growing culture. All that can be said at present is that some amount of calcium salt must be present in the culture during growth for good production of gelatinase. In the first paper of this series [Haines, 1931] it was shown qualitatively that a similar effect holds with regard to the enzyme attacking caseinogen, but it remains for future work to determine whether salts of calcium are essential for the formation of the true proteases which bring about degradation of the protein molecule.

Summary.

1. It is shown that, as measured by changes in viscosity, the gelatin-gelatinase system over the early stages of the reaction follows Schütz's law.

2. Values of a constant calculated from this relation are given for strains of *B. proteus*, *B. pyocyaneus*, *Pseudomonas*, *B. subtilis*, *Achromobacter*, *B. mesentericus* and a *Staphylococcus* grown in broth and for five of these organisms grown in a synthetic medium.

3. With all the five organisms salts of magnesium greatly stimulate growth in the synthetic medium without stimulating gelatinase formation, and salts of calcium stimulate gelatinase formation without affecting growth, the best effect being obtained in the presence of both salts.

4. By varying the concentration of the salts of calcium and magnesium all degrees of gelatinase formation from nil to the optimum may be obtained in the synthetic medium, with good growth in all cases.

5. The production of gelatinase in the synthetic medium with optimum salt concentration is quantitatively as good as in broth culture, and persists over a number of subcultures.

I am indebted to Dr G. S. Graham-Smith for his interest in this work and to Mr J. Barlow for experimental assistance.

REFERENCES.

Haines (1931). *Biochem. J.* **25**, 1851.
—— (1932). *Biochem. J.* **26**, 323.
—— (1933). *J. Hygiene.* In the Press.
Merrill and Clark (1928). *J. Bact.* **15**, 267.
Schütz (1885). *Z. physiol. Chem.* **9**, 577.
Stephenson (1930). Bacterial metabolism. (Longmans, Green and Co., London.)
Wilson (1930). *J. Bact.* **20**, 41.

LXVIII. TYROSINASE FROM THE SKIN OF CERTAIN BLACK RABBITS.

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(*Received February 28th, 1933.*)

Introduction.

The existence of tyrosinase in mammals has been reported on various occasions. Gessard [1903], for instance, claimed to have extracted it from a tumour in a horse. He gives, however, only the general outline and not the details of his experiments. Onslow [1915] made extracts from the skins of rabbits and mice. He obtained active extracts only if the animals were very young, from 2 to 4 days old. From black and from certain other coloured rabbits he obtained extracts which, on addition of hydrogen peroxide, formed a black pigment from tyrosine, as does tyrosinase without any addition of hydrogen peroxide. In accordance with the theory of Bach and Chodat [1903] current at that time, of the dual nature of oxidising enzymes, this would indicate that he had extracted the peroxidase component of the enzyme without a sufficiency of the "oxygenase" or peroxide component.

Hydrogen peroxide hastens the action of tyrosinase on tyrosine. This was first shown by Bach [1906] and by von Fürth and Jerusalem [1907]. It does not necessarily mean, however, that tyrosinase consists of a specific peroxidase and a peroxide. It might just as well mean, for instance, that tyrosinase exists in an oxidised and a reduced form, and that any suitable oxidising agent, by hastening its re-oxidation, will hasten its action. Hydrogen peroxide is not the only oxidising agent which hastens the action of tyrosinase on monohydric phenols. An *ortho*quinone does this likewise. A reducing agent, sulphite, retards its action [Pugh, 1930]. The dual constitution propounded by Bach and Chodat definitely does not apply to all oxidase systems. Suminokura [1930] has shown that laccase has not the constitution specific peroxidase-*plus*-peroxide, and the present author [1930] entirely failed to separate tyrosinase into such constituents.

In view of these considerations, the enzyme extracted by Onslow was of especial interest, as being probably ordinary tyrosinase, but from a mammalian source. Onslow reported occasional slight action on tyrosine without hydrogen peroxide, and sometimes slight darkening of the extract alone. This he interpreted by saying that he sometimes extracted a small amount of "natural peroxide" and "chromogen"; it might quite well have been merely a rather more concentrated tyrosinase and chromogen. As far as the present author is aware, however, no attempts which have been made hitherto to repeat Onslow's work have met with any success. The present attempts have met with a certain measure of success.

Experimental.

Koller [1930] showed that extracts from the skins of dominant black rabbits act on 3 : 4-dihydroxyphenylalanine very strongly in spite of addition of substances which inhibit the action of extracts from recessive black rabbits.

Onslow's black rabbits were recessive black rabbits. It was thought, therefore, that any black rabbits would serve the present purpose, and the early experiments were carried out with black rabbits from ordinary laboratory stock. In the first experiments, also, Onslow's procedure was not used, for the following reason. This procedure includes grinding up the skins with kieselguhr which contains a good deal of iron. Hydrogen peroxide in presence of ferrous salt oxidises phenol to catechol [Martinon, 1885]. To avoid iron, therefore, a porcelain press was used, and instead of passing the skins through a mincer, they were frozen with liquid air and broken up with pestle and mortar. Tyrosinase was not obtained. Next time kieselguhr was used, and half the product was extracted with ammoniacal water, half with phosphate buffer p_H 8·0. Still no tyrosinase was obtained (B). After that, Onslow's procedure was followed as closely as possible.

The young rabbits, 2–4 days old, were chloroformed and skinned. The skins were freed from subcutaneous fat and small blood vessels, washed, dried with filter-paper and weighed. They were then minced in a tissue mincer and ground up with chloroform water (1 cc. to every 2 g. skin) and kieselguhr to make a paste. The product was placed in muslin, and the liquid pressed out with the porcelain press, and finally filtered. To 2 cc. (or nearly) of the extract thus obtained were added (1) 0·5 cc. 0·2 % suspension of tyrosine in water and 0·2 cc. 0·05 % hydrogen peroxide, (2) 0·7 cc. water, and usually (3) 0·5 cc. tyrosine and 0·2 cc. water. Provided that the quantity of extract permitted, other tubes were also put up, containing 0·2 % *p*-cresol or guaiacol or catechol or 3 : 4-dihydroxyphenylalanine (dopa) in place of tyrosine, together with controls containing catechol and dopa, with water in place of extract. Each tube was made just alkaline to litmus by addition of sodium carbonate solution and after addition of a drop or two of chloroform was plugged with cotton-wool and incubated at 37°. The tubes were examined 12 hours later (as nearly as possible).

Extracts prepared in this way from black rabbits of the ordinary laboratory stock (6 litters of 30 animals in all; age 1–3 days) failed to give any pigment formation with tyrosine or *p*-cresol in presence of hydrogen peroxide; negative results were also obtained in the cases of two of the litters with tyrosine alone and in those of two others with guaiacol alone. On the other hand the extracts acted on catechol and dopa and therefore contained catechol oxidase; this enzyme has already been obtained by Wohlgemuth and his collaborators from the skins of various species including that of a white human being [Yamasaki, 1924].

Black Rex rabbits (3 litters of 17 animals in all; age 2–2½ days) were then tried. Of the first litter half died overnight just before they were to be used, so the two parts of this litter were put up separately. The extracts all gave negative results with tyrosine (both alone and with hydrogen peroxide); those prepared from the first litter were tested also on *p*-cresol with and without hydrogen peroxide but no pigment was formed. In all cases catechol oxidase was again present. The residues from the extractions in the case of the first litter were placed in phosphate buffer at p_H 8·0 and allowed to autolyse under toluene for a few months; after filtration the liquid was tested but no tyrosinase was found. (One of the residues from ordinary stock rabbits was allowed to autolyse similarly for a few weeks and again gave a negative result.)

I am indebted to Mr M. Pease for a pair of rabbits which provided several litters of recessive black young. He also killed and sent overnight two litters of similar young. The results are given in Table I. From the rabbits killed overnight no tyrosinase was obtained (A and B). It was noted by Onslow that freshness of material is essential. From some of the freshly killed rabbits

tyrosinase was obtained. It was, however, about 36 hours before the tyrosine darkened. The tubes with hydrogen peroxide in addition darkened a few hours earlier. In the last experiment from this pair of rabbits (III) the extract itself darkened slightly but distinctly after several days.

At this juncture the recessive black buck fell ill and was killed, and was replaced by another. The first litter died of the doe's neglect when about one day old, and was therefore dead for several hours before being put up. No tyrosinase was obtained (IV). Two subsequent litters contained some "dilute black" youngsters. The black of one litter were put up, but tyrosinase was not obtained (V). The experiments were then discontinued.

Table I.

Killed and sent overnight from Cambridge.

	No. of young rabbits	Age (days)	Tyrosine $+H_2O_2$	*p*-Cresol	*p*-Cresol $+H_2O_2$	Guaiacol $+H_2O_2$	Catechol
A	3	1	Nil	Nil	Nil	—	Darkened
B	5	2	Nil	Nil	Nil	Nil	Darkened

Born in Manchester.

	No. of young rabbits	Age (days)	Wt. of skins (g.)	Tyrosine	Tyrosine $+H_2O_2$	*p*-Cresol	*p*-Cresol $+H_2O_2$	Guaiacol	Guaiacol $+H_2O_2$	Catechol	Catechol $+H_2O_2$	Dopa
I	3	2½	35	Nil	Nil	Nil	Reddened	—	Nil	Darkened	—	Darkened
II	4	3	31	Darkened	Darkened	Nil	Nil	—	Nil	Darkened	—	—
III	2	3	26	Darkened	Darkened	Nil	Slowly orange	Nil	Nil	Darkened	Darkened	Darkened
IV	4	1	21	Nil	Nil	Nil	Nil	—	—	—	—	
V	3	3	27	Nil	Nil	—	—	—	—	—	—	

In the Table, "nil" with *p*-cresol indicates no reddening. A yellow colour was neglected, because blood alone was found sometimes to give a yellow colour with *p*-cresol. All the extracts in the present experiments were pinkish with a little blood, which came out of the skins when they were subjected to pressure. The appearance of reddening with *p*-cresol was therefore not easy to determine.

With regard to the use of kieselguhr, which might contaminate the skins with iron salts: first, the extracts contained no iron detectable with thiocyanate and hydrogen peroxide; and secondly, iron salts have no action on tyrosine in absence of hydrogen peroxide and absence of tyrosinase. Precautions were taken to ensure that the reagents and distilled water used were free from iron.

Discussion.

The fact that it has been possible to make an extract from any rabbit skins at all which, without addition of hydrogen peroxide, exhibits a tyrosinase activity, shows that mammalian tyrosinase does exist, and that it was almost certainly ordinary tyrosinase with which Onslow was dealing. The occurrence of tyrosinase in the present extracts was, however, much more rare than it appears to have been in Onslow's extracts. The present author has followed Onslow's procedure so closely that she is convinced that this difference is not a matter of technique, but some real difference in the skins undergoing extraction. The difference may be one of genetic constitution quite independent of the colour of the fur. The extracts in which tyrosinase could not be demonstrated, from animals of colours in which Onslow demonstrated tyrosinase, may either

not contain tyrosinase owing to some difference which prevents the tyrosinase from being extracted from the skins; or they may contain tyrosinase, together with some inhibitor which prevents the tyrosinase from acting, or some substance which prevents action on the substrate from becoming apparent by coloration.

The following observations suggest the possibility of the last alternative. (1) Onslow examined his test-tubes after 12 hours because he found that the colours tended to fade later. Now the grey colour produced by the action of dilute tyrosinase from mealworms on tyrosine does not fade on standing. There must, therefore, be something present in extracts from rabbit skins which tends to decolorise melanin. (2) The yellow colour produced by the action of tyrosinase on *p*-cresol and the red colour by action on tyrosine both tend to be decolorised by ordinary baker's yeast. When substrate, tyrosinase and yeast are mixed, coloration begins only at the surface of the liquid instead of throughout the liquid. If the colour is distributed throughout by shaking up, the colour then gradually fades from the body of the liquid, while coloration continues at the surface. (3) When extracts of individual pupae of white butterflies were being tested on *p*-cresol and on tyrosine, it was observed that the rate of coloration of *p*-cresol was not always paralleled by the rate of coloration of tyrosine. On one occasion extracts of two pupae took practically the same length of time to begin to colour *p*-cresol, and coloration proceeded at the same rate; yet the one extract began to colour tyrosine strongly in a few hours, the other showed no coloration after several days. The two pigments, from *p*-cresol and from tyrosine, appear not to be decolorised equally readily. Whether the explanation of the non-demonstrability of tyrosinase in some of the extracts from rabbit skins lies in decoloration or not, the possibility needs to be considered, together with non-extractability of the tyrosinase, or, alternatively, presence of true inhibitors.

From all the skins "catechol oxidase" was obtained. Tests in buffered liquids with controls showed that the coloration of catechol and of 3 : 4-dihydroxyphenylalanine (dopa) was not due merely to spontaneous autoxidation, but was truly hastened by the extracts. By "catechol oxidase" is meant an enzyme which acts on *o*-dihydric phenols, but not on the monohydric phenols, tyrosine, *p*-cresol or guaiacol. It is interesting to note that tyrosinase, from mealworms for instance, can be so diluted that it acts readily on the *o*-dihydric phenols catechol and dopa but not on tyrosine or *p*-cresol. It has not been proved in the present investigation that the "catechol oxidase" concerned is not merely dilute tyrosinase. It was reported previously [Pugh, 1930] that an extract of the mantle of the black slug *Arion ater* acts on catechol and dopa but not on *p*-cresol or tyrosine, and it was concluded that it contained catechol oxidase but not tyrosinase. It has since been found that pieces of solid mantle colour *p*-cresol distinctly though not tyrosine. Again this action can be imitated by suitably diluted tyrosinase from mealworms. Hence the mantle of *Arion ater* probably contains tyrosinase. Again, Dr F. C. Happold kindly provided some bacteria said to act on catechol but not on tyrosine. As grown and prepared by the present author they did indeed not act on tyrosine or *p*-cresol, but their action on catechol and on dopa was as weak as that of a diluted tyrosinase. If the existence of catechol oxidase is to be accepted as distinct from tyrosinase (as well as from dopa oxidase, which does not act on catechol), a preparation must be described which acts on catechol and on dopa at least as rapidly as does tyrosinase of such a strength that it acts readily on *p*-cresol and on tyrosine.

SUMMARY.

Attempts have been made to repeat the extraction of tyrosinase from the skins of young black rabbits by the procedure used by Onslow. The attempts failed both when the black rabbits were of unknown pedigree and when they were Black Rex. From known recessive black rabbits tyrosinase was extracted in some cases. This acted on tyrosine even in absence of hydrogen peroxide and was therefore ordinary tyrosinase. Catechol oxidase was obtained from all the skins.

I am greatly indebted to Prof. H. S. Raper for his helpful guidance and interest.

REFERENCES.

Bach (1906). *Ber. deutsch. chem. Ges.* **39**, 2126.
—— and Chodat (1903). *Ber. deutsch. chem. Ges.* **36**, 606.
von Fürth and Jerusalem (1907). *Beitr. chem. Phys. Path.* **10**, 131.
Gessard (1903). *Compt. Rend. Acad. Sci.* **136**, 1086.
Koller (1930). *J. Genetics*, **22**, 103.
Martinon (1885). *Bull. Soc. Chim.* (2), **43**, 155.
Onslow (1915). *Proc. Roy. Soc. Lond.* B **89**, 36.
Pugh (1930). *Biochem. J.* **24**, 1442.
Suminokura (1930). *Biochem. Z.* **224**, 292.
Yamasaki (1924). *Biochem. Z.* **147**, 203.

LXIX. NOTE ON THE SUPPOSED OXIDASE ACTIVITY OF COBALTAMMINES, WITH PARTICULAR REFERENCE TO TYROSINASE.

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(*Received February 28th, 1933.*)

Shibata and Shibata [1929] have shown that a number of complex salts of metals such as cobalt, nickel, copper, zinc and cadmium imitate in certain respects the action of oxidases and have put forward the view that oxidising enzymes are complex compounds containing metals such as these. As their substrate they used chiefly myricetin, an oxyflavone derivative of catechol. Now tyrosinase is specific in that it acts on certain monohydric phenols such as tyrosine and *p*-cresol, as well as on catechol. Laccase, which also acts on catechol, does not act on tyrosine, but it does act on guaiacol, which is not affected by tyrosinase. Peroxidase acts as laccase, but only in presence of hydrogen peroxide. It was therefore decided to test some of these complex salts on the distinguishing substrates, in order to find whether there are any such specific differences among these salts. Shibata [1929] distinguished only "oxidase" from "peroxidase" action.

The suggestion that oxidising enzymes contain certain metals has frequently been made since Bertrand [1897] came to the conclusion that laccase contains manganese. In particular, with regard to tyrosinase, Pinhey [1930] has suggested that tyrosinase contains iron, in order to account for the inhibition of its action by reagents such as cyanide. Now hydrogen peroxide in conjunction with ferrous salt is able to imitate the action of tyrosinase in certain respects. Martinon [1885] showed that this reagent is able to oxidise phenol to catechol, and Raper [1932] has shown that tyrosine can be oxidised to 3 : 4-dihydroxyphenylalanine by similar means. It has been shown by Pugh [1932] that ferrous salt in small quantity hastens the action of tyrosinase on certain monohydric substrates, a phenomenon which depends probably on the production of hydrogen peroxide during the action of tyrosinase. It would have been desirable, therefore, to use complex salts of iron in trying to imitate the action of tyrosinase; but as iron does not form such salts, cobalt salts such as those used by Shibata and Shibata have been investigated.

The salts in the following list were tested on the substrates named. In Series I, 0·5 cc. *M*/1000 solution in water of the salt being tested was added to each test-tube; in Series II, 0·5 cc. *M*/250 solution. In either Series, each salt was added to five test-tubes containing 2·5 cc. 0·2 % solution in water of the substrate named, except tyrosine, of which a saturated solution was used. To each of the five test-tubes was added 0·2 cc. water, except the fifth, to which 0·2 cc. 1 % hydrogen peroxide was added instead. For purposes of comparison, 0·5 cc. salt solution was added to 2·7 cc. water in a sixth test-tube.

Series I.

	Tyrosine	*p*-Creso	Catechol	Guaiacol	Guaiacol $+H_2O_2$
$[4NH_3CoCl_2]SO_4H$	Nil	Nil	Slightly orange → greyish	Nil	Slightly orange
*$[4NH_3CoCl_2]Cl$	Nil	Nil	Orange → greyish	Nil	Orange
†$[5NH_3CoCl]Cl_2$	Nil	Nil	Slightly yellowish	Nil	Nil
‡$[6NH_3Co]Cl_3$	Nil	Nil	Nil	Nil	Nil
*$[3NH_3Co(NO_2)_3]$	Nil	Nil	Yellow	Faintly orange	Faintly orange
*$[3NH_3Co\overset{Cl_2}{\underset{H_2O}{}}]Cl$	Yellow	Decolorised	Yellow	Orange-brown	Orange-brown
*$[5NH_3CoCO_3]NO_3$	Nil	Nil	Faintly yellowish → greyish	Nil	Slightly orange
$[4NH_3CoCO_3]NO$	Nil	Nil	Faintly yellowish → greyish	Nil	Slightly orange
$[5NH_3CoH_2O](NO_3)_2$	Nil	Nil	Very faintly greyish	Nil	Nil
$[4NH_3Co(H_2O)_2](NO_3)_2$	Nil	Nil	Very faintly greyish	Nil	Nil
$[5NH_3CoCrO_4]Cl$	Nil	Nil	Pinkish grey	Nil	Orange
$[4NH_3Co(CN)_2Co(CN)_4]$	Nil	Nil	Nil	Nil	Nil
$[5NH_3Co(Co(CN)_6)]$	Nil	Nil	Nil	Nil	Nil

Series II.

	Tyrosine	*p*-Cresol	Catechol	Guaiacol	Guaiacol $+H_2O_2$
$[4NH_3CoCl_2]SO_4H$	Nil	Nil	Slightly orange → greyish	(Several days) dirty green	Slightly orange → yellowish green
*$[4NH_3CoCl_2]Cl$	Nil	Nil	Yellow → green → greenish grey	Nil	Orange
†$[5NH_3CoCl]Cl_2$	Nil	Nil	Faintly greenish grey	Nil	Nil
‡$[6NH_3Co]Cl_3$	Nil	Nil	Nil	Nil	Nil
*$[3NH_3Co(NO_2)_3]$	Nil	Nil	Orange → green → greenish grey	Orange	Brownish orange
*$[3NH_3Co\overset{Cl_2}{\underset{H_2O}{}}]Cl$	Yellow	Decolorised	Yellow → green → grey	Orange-brown	Orange-brown
*$[5NH_3CoCO_3]NO_3$	Nil	Nil	Yellowish green → grey	Nil	Orange
$[4NH_3CoCO_3]NO_3$	Nil	Nil	Yellowish green → grey	Nil	Orange
$[5NH_3CoH_2O](NO_3)_2$	Nil	Nil	Faintly greyish	Nil	Nil
$[4NH_3Co(H_2O)_2](NO_3)_3$	Nil	Nil	Faintly greyish	Faintly orange	Faintly orange
$[5NH_3CoCrO_4]Cl$	Nil	Nil	Orange → greenish grey	Nil	Orange
$[4NH_3Co(CN)_2Co(CN)_4]$	Nil	Nil	Nil	Nil	Nil
$[5NH_3Co(Co(CN)_6)]$	Nil	Nil	Nil	Nil	Nil

The substances marked * are, according to Shibata, very active, the one marked † less active, that marked ‡ practically without action, as oxidases.

It is evident that the only one of the above salts which may be of interest with regard to tyrosinase is dichlorocobaltic chloride, $[3NH_3CoCl_2(H_2O)]Cl$. Now the action of this salt on the test substances may be an oxidative process similar to that brought about by tyrosinase or laccase, and accompanied by an absorption of oxygen; or the coloration may be due to some reaction between salt solution and substrate having no relation to the oxidations brought about by tyrosinase and laccase. It is to be noted that the colour produced with tyrosine is not the typical pink darkening to grey which would be expected with tyrosinase.

The action of this salt on tyrosine on the one hand, and on guaiacol on the other, was examined in Barcroft microrespirometers, in order to find whether the coloration is accompanied by oxygen absorption or not. The left-hand bottle contained 1 cc. substrate solution, 1·5 cc. water, 0·5 cc. $M/250$ salt solution. The right-hand bottle contained 2·5 cc. water, 0·5 cc. salt solution. When, in addition, 0·2 cc. strong caustic potash was placed in the small tube fused into each bottle, no indication of oxygen uptake was obtained at all, with either tyrosine or guaiacol as substrate.

Tyrosine.	Exp. 1.	2 hr.,	10	mm.³ O_2;	23 hr.,	11	mm.³ O_2
	,, 2.	3 ,,	0	,,	22 ,,	−6·5	,,
	,, 3.	3 ,,	2	,,	21 ,,	−6	,,
Guaiacol.	Exp. 1.	2 hr.,	0	mm.³ O_2;	23 hr.,	−4	mm.³ O_2
	,, 2.	3 ,,	1	,,	22 ,,	3	,,
	,, 3.	3 ,,	3	,,	21 ,,	−1	,,

When no potash was present, there appeared to be a slight but definite "oxygen absorption," and the amount of this "absorption" appeared to vary with the concentration of salt used. These "absorptions," however, were very irregular, the manometer sometimes reaching the same level again on both sides.

Tyrosine.	Exp. 1.	2 hr.,	12	mm.^{3} O_2;	3 hr.,	12	mm.^{3} O_2
	,, 2.	1 ,,	9	,,	3 ,,	2	,,
	,, 3.	1 ,,	18	,,	20 ,,	27	,,
Guaiacol.	Exp. 1.	2 hr.,	16	mm.^{3} O_2;	3 hr.,	16	mm.^{3} O_2
	,, 2.	1 ,,	18	,,	3 ,,	24	,,
	,, 3.	1 ,,	12	,,	20 ,,	9	,,

The following experiments were carried out in presence of potash, using different concentrations of salt solution. The maximum deviation from zero is given.

Salt concentration	Tyrosine	Guaiacol
M/125	−5 mm.^{3} at $\frac{1}{2}$ hr.	3 mm.^{3} at 19 hr.
M/250	5 ,, 22 ,,	4 ,, $\frac{1}{4}$,,
M/500	10 ,, 5 ,,	−5 ,, 19 ,,
M/1000	Nil	−5 ,, $\frac{1}{4}$,,

In absence of potash the following results were obtained.

Salt concentration	Tyrosine					
M/125	2 hr.,	18	mm.^{3} O_2;	24 hr.,	18	mm.^{3} O_2
M/250	,,	13·5	,,	,,	9	,,
M/500	,,	5·5	,,	,,	0	,,
M/1000	,,	4	,,	,,	0	,,
	Guaiacol					
M/125	5 hr.,	17	mm.^{3} O_2;	23 hr.,	24	mm.^{3} O_2
M/250	,,	16	,,	,,	17	,,
M/500	,,	8	,,	,,	3	,,
M/1000	,,	7	,,	,,	6	,,

The failure of this "oxygen absorption" to occur in presence of potash in the small tube, which merely absorbs carbon dioxide from the air in the bottle, is difficult to explain if it really indicates oxygen absorption. It seems more probable that any oxygen absorption which may occur is too small to measure by these means, and that the apparent slight absorption in absence of potash is due to some other cause.

The action of dichrocobaltic chloride on catechol was accompanied by a definite and measurable oxygen absorption, whether in presence or absence of potash.

	With KOH			Without KOH		
	1 hr.	3 hr.	20 hr.	1 hr.	3 hr.	25 hr.
Exp. 1	43	63	90	28	35	49
,, 2	41	59	86	30	36	40

Catechol alone during this time absorbed no measurable oxygen.

The respirometer experiments provide no evidence that the coloration of monohydric phenols by dichrocobaltic chloride is due to oxidation. Attempts were therefore made to carry out the reactions in Thunberg tubes from which air had been removed by washing out with nitrogen. It was found, however, that tyrosinase from mealworms still coloured *p*-cresol under nitrogen freed from oxygen (1) by passing through several wash-bottles containing alkaline pyrogallol, and (2) by passing through a furnace containing copper. This indicated that these methods did not remove oxygen sufficiently completely for

the present purpose. Eventually the procedure of Kautsky and Thiele [1926] was found satisfactory.

The nitrogen was obtained from a cylinder and passed through a pressure gauge and regulator. To remove oxygen, the gas was then passed through a mixture of sodium hydrosulphite and caustic potash in an inverted aspirator. By means of a Berkefeld filter it was broken up into fine bubbles as it entered the liquid. The outlet from the aspirator was covered with a disc of lead, and glass tubing passed through a hole in the disc. The gas was then washed through 10 % KOH and finally water. The glass ends were joined together with lengths of flexible lead tubing. All the lead-glass junctions were made gas-tight with white sealing-wax. Each Thunberg tube in turn was sealed through lead tubing and white sealing-wax to a three-way tap, which led on past a two-way tap (which ended the gas-tight section) to an oil-pump and mercury manometer. Each Thunberg tube was alternately evacuated and filled with oxygen-free nitrogen at least four or five times.

Every liquid which was to be used was boiled for 5–10 minutes on the previous day and placed to cool in a desiccator over pyrogallol solution into which alkali was spilt after the desiccator had been evacuated. The salt was freshly dissolved in water thus prepared. The tyrosinase solution alone was not boiled but was allowed to stand in the anaerobic desiccator for a day before use.

At the beginning of each series of experiments, and again at the end, a tube was put up containing 2·5 cc. 0·2 % *p*-cresol in phosphate buffer p_H 6·5 and 0·5 cc. tyrosinase, and another containing buffer and tyrosinase. The enzyme was contained in a small tube placed inside the Thunberg tube and was mixed with the liquid outside it after the Thunberg tube had been filled with nitrogen. Absence of coloration of the *p*-cresol indicated that the apparatus was efficient. In the experimental series, 0·5 cc. *M*/250 dichrocobaltic chloride solution in water was placed in the inner tube, and 2·5 cc. either 0·2 % catechol or guaiacol or *p*-cresol or saturated tyrosine solution in water in the Thunberg tube. In one tube was placed plain water and salt. Corresponding to each tube, a parallel tube was put up in air. Two separate series of experiments agreed in yielding the following results.

	In air	In nitrogen
Catechol	At once green → dirty green	Green for an instant → slightly yellowish → pinkish → nil
Guaiacol	Orange → purplish brown deposit	Orange → purplish brown deposit. (Coloration less than in air)
p-Cresol	Colourless or slightly yellowish	Colourless or slightly yellowish
Tyrosine	Yellow → slightly pinkish yellow	Yellow → slightly pinkish yellow
Water	[(Purplish) pink] → yellow	[(Purplish) pink] → yellow

When air-free solutions were used, in air, the green colour with catechol did not persist as when the liquid was saturated with air. The green colour faded to yellowish until the tube was shaken up, when it reappeared at once, only to fade again on standing. This could be repeated a number of times before the green colour became permanent. It is evident that the green colour with catechol requires oxygen. There is some indication that the coloration of guaiacol may need oxygen, but this is not certain. As for tyrosine and *p*-cresol, there is no evidence that oxygen is required for the production of the change in colour, or that any oxidative action takes place at all. The colour effects on tyrosine and *p*-cresol are due probably to some cause other than oxidation.

(It was shown similarly that oxygen is required for the production of the green colour by *M*/250 1 : 6-dichlorotetrammine cobaltic chloride and by *M*/250 trinitratotriammine cobalt with catechol.)

On standing, dichrocobaltic chloride solution itself changed in colour from purple to yellow. The orange-yellow colour which appeared with tyrosine and the change in colour with *p*-cresol were, however, very much more rapid. But addition of alkali to the salt solution caused it to become yellow rapidly, and addition of acid to the yellow liquid restored the purplish pink colour. Addition of acid to the yellow liquid from tyrosine did not restore it to purplish pink. Alkali added to the yellow from tyrosine caused a change from dull to slightly brighter yellow. Acid added to the colourless liquid from *p*-cresol caused no change, alkali produced a bluish colour turning green. It is evident that the colour changes with tyrosine and *p*-cresol are not due to the effect of p_H changes on the salt solution. The reactions could not be carried out in buffered solution, as the salts were thereby rendered inactive[1].

Finally, to determine whether dichrocobaltic chloride has any action on *p*-cresol analogous to that of tyrosinase, the two former substances were mixed (1) in presence of glycine and (2) in presence of aniline. If an *ortho*quinone had been produced by the action of the salt on *p*-cresol as by the action of tyrosinase, a red colour would have been expected in presence of glycine and a red precipitate of anilinoquinone in presence of aniline. In the glycine series 4 cc. 1 % *p*-cresol solution in water were placed in each of three test-tubes. To the first was added 1 cc. 0·75 % glycine solution in water, to the second 1 cc. glycine solution diluted 1 in 10, to the third water. A fourth test-tube contained 4 cc. water and 1 cc. 0·75 % glycine, a fifth tube 5 cc. water. To each test-tube were added 2 cc. *M*/250 salt solution. The first two tubes, containing glycine with *p*-cresol in addition to salt, developed a slightly pinkish tinge relative to the third tube, even when the glycine was alkaline. But the fourth tube, containing glycine with salt, retained a (yellowish) purple colour even when the fifth tube had become deep yellow. The pinkish colour is therefore due to some effect of the glycine on the salt even in absence of *p*-cresol and provides no evidence of the production of an *ortho*quinone.

In the aniline series, 4 cc. 1 % *p*-cresol solution in water were placed in each of three test-tubes. To the first and second was added 1 cc. saturated aniline water, to the third water. A fourth tube contained 4 cc. water and 1 cc. aniline water, a fifth tube 5 cc. water. To each test-tube were added 2 cc. *M*/250 salt solution except to the second, to which 2 cc. water were added. The fourth tube at once became orange, finally brown. The first tube acquired a pinkish yellow tinge and deposited a slight sediment next day. The second tube remained colourless, the third tube became faintly yellowish. This seems to indicate that the cobalt salt has indeed a slight action, but it is probably similar to that of ferrous salt, needing a trace of hydrogen peroxide formed by the action of air on the salt solution. Addition of 2 drops of 1 % hydrogen peroxide to a mixture as in the first test-tube resulted in a rapid production of reddish brown sediment.

The change of salt solution from purple to yellow took place under nitrogen, as stated, and was hastened by alkali. It was, however, also hastened by

[1] Dr S. H. C. Briggs has kindly given me the following information. A freshly prepared concentrated solution of dichrocobaltic chloride in water is bluish green (or greenish blue). The purple colour of the dilute solutions used in all the present tests is probably due to the presence of cherry-red triaquotriammine cobaltic chloride, $[3NH_3Co3H_2O]Cl_3$, formed by replacement of Cl by H_2O, a process undergone very readily by dichrocobaltic chloride. The further change in colour to yellow is probably due to hydrolysis, H_2O in the complex being replaced by OH. This explains the action of NaOH in deepening the yellow colour, and the regeneration of the purple tint on addition of acid.

hydrogen peroxide. When 1 cc. 1 % hydrogen peroxide was added to 1 cc. *M*/250 salt solution, profuse evolution of bubbles took place; the liquid changed to yellow and eventually deposited a yellowish brown sediment.

Although dichrocobaltic chloride acts as a catalase, 1 : 6-dichlorotetrammine cobaltic chloride and trinitratotriammine cobalt do not.

In conclusion, it may be said that no evidence has been found which would suggest that the action of tyrosinase can be imitated by any of the complex metallic salts tested. The change in colour of tyrosine and *p*-cresol solutions by dichrocobaltic chloride is not a process which requires oxygen. The coloration of catechol by dichrocobaltic chloride, 1 : 6-dichlorotetrammine cobaltic chloride and trinitratotriammine cobalt is however a process requiring oxygen.

Summary.

A number of cobaltammines have been tested on the substrates which distinguish certain oxidases from one another with a view to finding whether any of them imitate the action of tyrosinase. None of those tested was found to do so.

The action of several of the cobaltammines on catechol was shown to require oxygen.

I am greatly indebted to Dr S. H. C. Briggs, for his kindness in providing the cobaltammine salts needed for this investigation and to Prof. H. S. Raper for his encouragement and advice.

REFERENCES.

Bertrand (1897). *Compt. Rend. Acad. Sci.* **124**, 1355.
Kautsky and Thiele (1926). *Z. angew. Chem.* **152**, 342.
Martinon (1885). *Bull. Soc. Chim.* (2), **43**, 155.
Pinhey (1930). *J. Exp. Biol.* **7**, 19.
Pugh (1932). *Biochem. J.* **26**, 106.
Raper (1932). *Biochem. J.* **26**, 2000.
Shibata (1929). *Acta Phytochimica*, **4**, 373.
—— and Shibata (1929). *Acta Phytochimica*, **4**, 363.

LXX. THE GLYOXALASE ACTIVITY OF THE RED BLOOD CELL.

THE FUNCTION OF GLUTATHIONE.

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(*Received March 2nd, 1933.*)

In view of the importance of methylglyoxal as an intermediate product in carbohydrate metabolism, the study of the transformation of methylglyoxal into lactic acid and of the kinetics of glyoxalase activity has attracted much attention. The subject has acquired a new interest by reason of Lohmann's finding [1932] that glutathione[1] acts as a co-enzyme for the glyoxalase present in tissue extracts.

Investigations so far, however, have been carried out almost entirely with tissue extracts and minced tissues, and little work has been done with intact cells. This paper presents a quantitative study of such cells.

The cells studied are human red blood corpuscles. The work follows an observation made by one of us (J. H. Q.) two years ago that the glyoxalase activity of the red blood cell disappears on complete lysis of the cell by considerable dilution with distilled water. The reason for this did not become apparent until work was commenced on the influence of thiol groups on carbohydrate breakdown, and Lohmann's observation [1932] threw light on the phenomenon.

The experiments recorded below present a number of points of interest. They not only show that the disappearance of glyoxalase activity of the red blood cell by lysis with water is simply due to the dilution of the glutathione of the cell, but they provide evidence that the transformation of methylglyoxal into lactic acid proceeds *via* the intermediate formation of a compound between methylglyoxal and glutathione, this compound breaking down rapidly into lactic acid in presence of glyoxalase. Observations of glyoxalase activity of the red cell under aerobic and anaerobic conditions show an interesting parallelism with the well known effects of such conditions on glycolysis (the Pasteur reaction), and the significance of this parallelism is discussed.

Experimental methods.

Measurements of the transformation of methylglyoxal into lactic acid were made by the manometric method of Warburg [1926]. Formation of lactic acid in a Ringer solution buffered with CO_2 and $NaHCO_3$ gives rise to evolution of CO_2.

Blood was obtained from a number of male subjects. The cells from the fresh defibrinated blood were usually washed three times on the centrifuge with about

[1] The name glutathione is reserved in this paper for the reduced substance (GSH). The oxidised form (GSSG) is referred to as oxidised glutathione.

an equal volume of saline. (When the cells are described as "well washed," 5 volumes of saline were used in the 3 washings.)

The suspension of washed cells was made up to 20 times its volume with Locke solution (NaCl, KCl, $CaCl_2$), and 0·3 cc. (usually) of the diluted suspension was placed in the manometric vessel. Locke solution and 0·16 M $NaHCO_3$ were then added to make a total volume of (usually) 2·9 cc., and 0·3 cc. of a methylglyoxal solution (diluted with Locke solution and neutralised with $NaHCO_3$) was placed in the side-bulb of the vessel. The vessel was then flushed out with a gas mixture containing CO_2 and N_2 or O_2, and after its contents had been equilibrated at 37° the methylglyoxal was added to the dilute cell suspension.

The output of gas during the first 3 to 5 minutes is not a measure of the velocity of the reaction during that period. Small gas changes may take place owing to the contents of the side-bulb not being exactly adjusted in $NaHCO_3$ or CO_2 concentrations. More important is the lag in the manometric output of the CO_2 formed, due to the necessity for a certain excess CO_2 content to be built up in the solution, the excess depending on the rate of the chemical reaction and on the rate of shaking. After about 5 minutes the rate of CO_2 output appears to correspond with the rate of the chemical reaction.

The $NaHCO_3$ concentration was initially 0·025 M, and the gas mixtures contained 4·5–5 % CO_2. The initial p_H was therefore about 7·45 to 7·50; during the reaction the p_H falls. Under our conditions a CO_2 output of 350 mm.^{3} will cause a fall in p_H of 0·25–0·31. Retention of CO_2 by the solutions due to the carbonate-bicarbonate buffer solution may be calculated, in a similar way to that used by Jowett [1932], to be about 1·5%. Rough measurements have shown that per mg. of glutathione added to the solutions there is likewise a retention of 1–2 %. As the retentions are small, they have usually been ignored in our calculations.

The concentration of red blood cells in the concentrated suspensions was variable. In order to calculate the gas output in terms of mg. dry weight of cells employed, the dry weight of a known volume of the original concentrated suspension has to be determined.

The glyoxalase activity of the intact cells under anaerobic conditions.

The rate of formation of lactic acid by intact cells is shown, in Fig. 1, as a function of the CO_2 output. The curve shows the whole course of the reaction, which proceeds until all the methylglyoxal has been transformed. The velocity of the reaction is constant until the methylglyoxal concentration has fallen considerably, and it appears therefore that the enzyme system is saturated with methylglyoxal for the major part of the reaction. Analysis of a number of such curves showed that the methylglyoxal concentration (reckoned in terms of the CO_2 output still to come) is about $5 \times 10^{-4} M$ when the velocity has fallen to one-half its original value.

In Table I are given measurements of the rate of CO_2 output of cells in a Ringer solution saturated with the $N_2 + CO_2$ gas mixture. The rate ($Q_M^{N_2}$) is stated in terms of the number of mm.^{3} of CO_2 evolved per hour per mg. dry weight of cells, and the measurements relate to the initial portion of the velocity curve where the rate is constant.

The glyoxalase activity of cells from blood freshly drawn and defibrinated is evidently similar to that of cells freed from serum by washing with saline. Further washing with saline, or keeping the cells 2 hours in Ringer solution at

37° in presence of nitrogen, does not affect their activity appreciably. On keeping the cells 1–2 days at 0°, their activity is little affected when there is some access to oxygen.

Comparison with measurements made by Warburg, Posener and Negelein [1924] on two other tissues (rat liver and carcinoma) by the manometric method

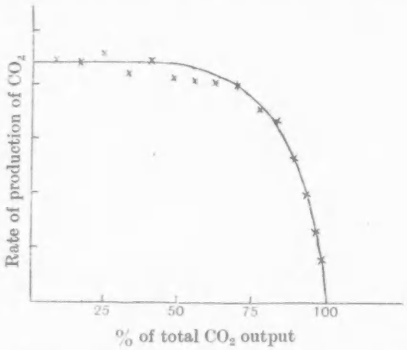

Fig. 1. Rate of glyoxalase action of human red blood cells.

Table I.

A. *Glyoxalase activity of human red blood cells.*

In Ringer solution at 37° $N_2+5\%$ CO_2. Initial methylglyoxal conc. = 0·004 to 0·01 *M*
Dry weight of cells = 4–5 mg.

	$Q_M^{N_2}$
Whole blood, freshly drawn	71*
Red cells, washed with saline	51, 73
Red cells, washed with saline†	49, 74, 72
Red cells, well washed with saline†	56, 52

B. *Changes in activity of human red blood cells.*

At 37° in N_2+CO_2.	% change
(*a*) In Ringer solution 2 hours	−7
(*b*) In glucose-Ringer solution 1½ hours	−1
At 0° with some access to oxygen.	
(*a*) Strong cell suspension, kept 2 days	0
(*b*) Dilute suspension in Locke, kept 1 day	+2

C. *Glyoxalase activity of other tissues.*

	$Q_M^{N_2}$
Rat liver	24
Rat carcinoma	29

* Assuming the dry weight of red cells in 1 cc. of blood to be 160 mg.
† The cells were kept 2 hours in the Ringer solution at 37° in N_2+CO_2 before adding the methylglyoxal.

shows that the glyoxalase activity of human red blood cells is high. The measurements already given appear to show that we are dealing with a fairly stable enzyme system of reasonably constant activity.

The glyoxalase activity of lysed cells.

When the red cells are completely lysed their glyoxalase activity practically disappears. This is shown by the data in Table II. The cells were lysed by adding 1 volume of a concentrated cell suspension (previously washed with saline) to 20 volumes of water. With this large volume of water, the cell structure appears to be rapidly broken up: after adding Ringer solution we could detect no cell fragments microscopically, and only stromata could be centrifuged down. When the original cell contents are diluted about 200 times in all, the rate of CO_2 output with methylglyoxal is only some 2 % of that shown by intact cells[1].

Table II. *Effects of lysis on glyoxalase activity.*

Lysed cells in presence of Ringer solution and N_2 + 5 % CO_2	mm.^{3} CO_2 evolved per min.
Intact cells*	6·53, 4·90, 6·17, 4·84, 5·78
Lysed cells*	0·14, 0·10, 0·16

* 0·015 cc. of the original concentrated cell suspension was diluted 20 times with Ringer solution, or with water (to effect lysis); it was then diluted 10 times again with Ringer solution before adding methylglyoxal.

Now Lohmann has shown that glutathione acts as a co-enzyme for the glyoxalase of muscle- and liver-extracts. If glutathione is a co-enzyme for the glyoxalase of red cells, lysing the cell and diluting its contents may lower the glutathione concentration of the cell to a point where it cannot appreciably assist in transforming methylglyoxal into lactic acid.

We have therefore made experiments in which varying amounts of glutathione were added to the diluted lysed cells.

Table III. *Effect of glutathione on glyoxalase activity of lysed red cells.*

Exp.	Rates of CO_2 output in nitrogen.	mm.^{3} CO_2 evolved per min.
1	Intact cells	6·53
	Intact cells + 0·031 % GSH	6·30
	Lysed cells	0·14
	Lysed cells + 0·031 % GSH	5·23
	(Methylglyoxal + Ringer alone)	0·02
2	0·028 % GSH	0·06
	Lysed cells	0·10
	Lysed cells + 0·028 % GSH	4·87
3	Intact cells	6·51
	Lysed cells + 0·008 % GSH	2·14
	Lysed cells + 0·016 % GSH	3·26
	Lysed cells + 0·039 % GSH	5·14
4	Lysed cells	0·11
	Lysed cells + 0·008 % GSH	1·83
	Lysed cells + 0·023 % GSH	4·15
	Lysed cells + 0·062 % GSH	6·49
5	Lysed cells + 0·047 % GSH	6·33
	Lysed cells (heated) + 0·047 % GSH	0·03

The data given in Table III show that moderate concentrations of glutathione restore glyoxalase activity to the system, and glutathione is evidently function-

[1] The small quantity of material taken and the extensive dilution are to be noted. Warburg and Christian [1931], who report that the red cell contents show considerable glyoxalase activity, appear to have used more material less extensively diluted.

ing as a co-enzyme. Detailed results are given in Table IV. Methylglyoxal alone, or methylglyoxal and glutathione together, show no appreciable output of gas—the observed figures are hardly outside the experimental error. When the lysed cells are heated at 100° for 2 minutes before adding to methylglyoxal and glutathione, no appreciable gas output takes place: the gas output is almost entirely due to the action of a thermolabile enzyme.

Table IV. *Effect of glutathione on the glyoxalase activity of lysed cells.*

Gas mixture $N_2 + 5\ \%\ CO_2$.

Each vessel contains 0·015 cc. of a concentrated cell suspension, diluted or lysed, together with Ringer solution.

In each side-bulb are 1·1 mg. methylglyoxal in 0·2 cc. Ringer solution. The side-bulbs also contain varying quantities of glutathione in Ringer solution. The volume of liquid in each vessel after adding the contents of the side-bulb is 3·2 cc. Vessel constants (k_{CO_2}) 1·2–1·5.

	Intact cells	Lysed cells		
cc. 0·5 % GSH	0	0·05	0·1	0·25
Time in mins.	Output of CO_2 in mm.³			
6	44	6	11	16
13	89	19	30	44
20	135	33	51	78
27	181	49	74	114
34	225	63	97	150
41	263	78	119	186
48	300	93	142	222
55	330	107	164	254
62	347	121	186	284
69	356	134	206	310

As was found by Lohmann, the activity does not rise as rapidly as the glutathione concentration. Experiments such as that shown in Table V show that the activity has nearly reached a limiting value at a glutathione concentration of about 0·06 %, further increase in glutathione concentration having little effect.

Table V. *Volume of CO_2 produced from lysed cells in presence of methylglyoxal and GSH. Comparison with intact cells (same weight).*

	mm.³ CO_2 evolved in first 30 mins.
Intact cells alone	283
Lysed cells + GSH (0·031 %)	278
Lysed cells + GSH (0·062 %)	350
Lysed cells + GSH (0·094 %)	370

Now the glutathione content of the human red blood cell is about 0·13 %[1], and it would therefore be expected that the cells should be showing their maximum glyoxalase activity. If so, this maximum activity is less than can be shown by the enzyme when set free from the cell (Table V). We do not however wish to lay stress on this point, since we have not yet succeeded in understanding fully the kinetics of the transformation in presence of lysed cells and added glutathione. The rate of gas output is seldom constant for more than a short part of the reaction.

[1] The figure is based on the finding of King, Baumgartner and Page [1930] that the glutathione content of blood is 0·06 %, and on the fact that all the glutathione is present in the cells.

We wish rather to stress the facts, (1) that the glyoxalase activity of the intact cells is of the same order as that of lysed cells to which glutathione has been added, and (2) that the glutathione concentration necessary to activate the free enzyme completely is very similar to the glutathione concentration of the intact cells. These two facts support very strongly the view that glutathione is a co-enzyme for the glyoxalase of the red blood cell.

Further, since the enzyme so extensively diluted can have its original activity restored by addition of glutathione alone, it is probable that no other soluble co-enzyme is concerned in glyoxalase action.

We will at this point present evidence that the manometric outputs of gas observed in our experiments are equivalent to the lactic acid formed. The comparisons are shown in Table VI. The manometrically observed CO_2 outputs are corrected for a slight retention of CO_2 as previously described.

Table VI. *Comparison of gas output with lactic acid formation.*

	Methylglyoxal added to	Retention factor	Corrected CO_2 output mm.[3]	mg. lactic acid formed	
				Calculated	Observed
1	Intact cells (N_2, CO_2)	1·017	348	1·40	1·41
2	Intact cells (O_2, CO_2)	1·017	347	1·40	1·41
3	Lysed cells + GSH (N_2, CO_2)	1·035	340	1·37	1·39
4	Lysed cells + GSH (O_2, CO_2)	1·035	316*	1·28*	1·32

* These figures may be slightly too low owing to oxidation of GSH resulting in oxygen uptake.

Chemical determinations of the amount of lactic acid formed (corrected by suitable blank determinations) were made by the method of Friedemann, Cotonio and Shaffer [1927]. It will be seen that within the experimental error the gas output represents entirely formation of lactic acid.

The combination of methylglyoxal and glutathione.

Lohmann [1932] has obtained some evidence that glutathione combines with methylglyoxal in weakly alkaline solutions. A precipitation test for methylglyoxal was the means employed. Such a combination would be of significance for the mechanism of action of glutathione, and we have therefore sought to confirm Lohmann's finding. This has been done by three independent methods.

(1) When glutathione and methylglyoxal are mixed together in Ringer solution at p_H about 7·5, a rapid but not instantaneous evolution of CO_2 takes place (Table VII). The evolution is both greater and more rapid the higher the methylglyoxal concentration, and may be most reasonably interpreted as consequent on a combination of methylglyoxal and glutathione. If the product is a stronger acid than is glutathione, evolution of CO_2 from the system would be expected. On this view, the strengthening of the acidity is not great, only about 1/10 mole of acid being set free per mole of glutathione. The data obtained are not sufficiently accurate for a really quantitative analysis; nevertheless, a rough analysis of the velocity curves suggests that the combination is not a straightforward bimolecular process followed by CO_2 output proportional to the amount of combination[1].

[1] In experiments on glyoxalase activity in which several mg. of glutathione are added, a gas exchange due to formation or breakdown of the compound will affect our interpretation of CO_2 output as due entirely to lactic acid production. The error is too small to affect our conclusions.

(2) More direct and simpler evidence of a combination is afforded by iodine titrations carried out (Table VII) on acid solutions of glutathione which have been kept some hours at 37° in presence of methylglyoxal. The presence of methylglyoxal has lowered the iodine titre[1], and this gives direct evidence of a combination of the sulphydryl group of glutathione with methylglyoxal. Under the conditions cited (Table VII) the combination of methylglyoxal has occurred

Table VII. *Evidence of reversible combination between methylglyoxal and GSH.*

(*a*) *Manometric.*

Volume of CO_2 produced by admixture of methylglyoxal and GSH in $NaHCO_3$ saline.

Concentration of methylglyoxal (*M*)	Concentration of GSH (*M*)	Time required to reach 50 % completion of output (min.)	Total moles of CO_2 produced
0·0125	0·0057	3½	0·00059
0·0094	0·0057	4	0·00056
0·0062	0·0057	6	0·00047

(*b*) *Iodimetric.* Solutions of 4 cc. kept 4 hours at 37° at p_H 3·0.

Iodine (*N*/250) titrations in cc.

	Direct titre	Total titre after adding $NaHCO_3$
1. GSH (6 mg.)	4·65	4·70
2. GSH (6 mg.) + methylglyoxal (0·96 mg.) ...	3·05	5·90

Theoretical difference between direct titres for complete combination = 3·31,

$$\therefore \%\ \text{combination of methylglyoxal} = \frac{1{\cdot}60}{3{\cdot}31} \times 100 = 48.$$

to the extent of 48 %—assuming that one molecule of the glyoxal has combined with one molecule of glutathione[2]. It is suggested that the compound formed is probably $CH_3.CO.CHOH.SG$. Dissociation of the compound is accelerated by the addition of sodium bicarbonate, as shown by the increased iodine titre after such addition. Actually the increased titre is greater than that required for the total —SH present and perhaps indicates that under these circumstances some of the glyoxal undergoes oxidation by the iodine.

(3) A third line of evidence follows from the velocity-output curve of the glyoxalase action of red blood cells in presence of glutathione.

Fig. 2 shows that the presence of moderate amounts of glutathione in the Ringer solution bathing the cells does not affect very appreciably the initial rate of gas output (compare Curve I with Curves II and III). In the presence of added glutathione, however, the rate falls off earlier, and the more so the greater the amount of the glutathione present (Curves II, III and IV). The total gas output is the same in all cases. These results may be interpreted as due to the formation of a compound between glutathione and methylglyoxal, the compound being unable to penetrate the cells. The rate is then dependent on the concentration of free methylglyoxal, which is, of course, lower when glutathione has been added, and hence glutathione brings about a falling off in rate at an earlier stage of the reaction. We have evidence that the compound decomposes

[1] A similar result has previously been found by Kühnau [1931].

[2] Thiolacetic acid under similar conditions combines with methylglyoxal to the extent of over 90 %. Reversal is effected by the addition of $NaHCO_3$.

again in the fact that the total gas output is the same in all cases, and evidence of the buffering effect of the decomposition of the compound on the methylglyoxal concentration in the less abrupt falling off in rate when glutathione is present. The curves are therefore definitely in agreement with the view that glutathione and methylglyoxal combine reversibly and that their compound cannot penetrate the red cells.

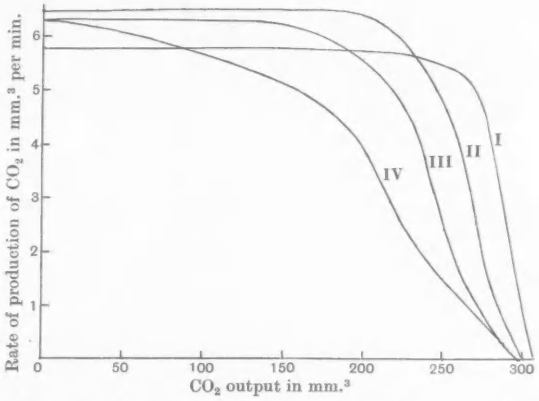

Fig. 2. Effect of glutathione on glyoxalase activity of human red blood cells.

Curve	mg. GSH in 1·67 cc.	$\frac{[GSH]}{\text{Initial [methylgloxal]}}$
I	0	0
II	0·75	0·18
III	1·50	0·36
IV	3·00	0·72

It is unlikely on *a priori* grounds that such a compound could penetrate red cells, since it is believed that glutathione cannot do so. This belief is based on the fact that no glutathione is found in serum: a partition could hardly favour the cells so strongly. The belief receives support from the fact (Table I) that washing cells with saline and shaking them in Ringer solution for 2 hours has little effect on their glyoxalase activity, for if a fair proportion of the glutathione could diffuse out of the cells, the glyoxalase activity should fall considerably.

It is reasonable to suppose, on the evidence given, that a compound between methylglyoxal and glutathione is formed, and since the latter acts as a co-enzyme for the transformation of methylglyoxal into lactic acid, it is probable that the action of the glutathione is exerted through the formation of the compound.

The scheme of reaction which we suggest then as actually taking place and which is catalysed by glyoxalase is as follows:

$$CH_3.CO.CHO + GSH \rightleftharpoons CH_3COCHOH.SG \xrightarrow{H_2O} CH_3CHOHCOOH + GSH.$$

The effects of oxygen on the glyoxalase activity of red cells.

So far we have considered experiments made under anaerobic conditions, *i.e.* with only traces of oxygen present. We now turn to experiments on the effects of exposure to oxygen on the glyoxalase activity of the red blood cells.

The data in Table VIII show the relative rates of glyoxalase activity when cells are shaken for varying times in Ringer solution at 37° before the methylglyoxal is added, (*a*) in a nitrogen-carbon dioxide mixture, (*b*) in an oxygen-carbon dioxide mixture. The methylglyoxal is added, as usual, from the side-bulb of the vessels.

The rate of glyoxalase activity is lower in oxygen than in nitrogen. When the cells are kept in oxygen for periods up to an hour, the rate is about 80 % of that in nitrogen. With longer prior exposure to oxygen, the relative rate in oxygen falls off further. In the last two experiments recorded in Table VIII

Table VIII. *Action of O_2 on glyoxalase activity of intact cells. Relative rates in N_2 and O_2.*

Exp.	Rate in N_2+5 % CO_2	Rate in O_2+5 % CO_2	Time of exposure of cells in Ringer solution at 37° (min.)	
1	100	80	41	
2	,,	82	61	
3	,,	71·5	124	
4	,,	69·5	128	
5	,,	77	121	
6	,,	55	121	
7	,,	44	88	Cells well washed
8*	,,	29	120	with saline

* For details of this experiment see Table XII.

the cells were more thoroughly washed with saline before use than was done in the other experiments: the effect of more thorough washing is to increase the falling off in glyoxalase activity which takes place in presence of oxygen.

The experiments of Table IX show that the presence of 0·2 % glucose in the Ringer solution diminishes the effect of oxygen on the glyoxalase activity. After 2 hours' shaking in Ringer solution in oxygen before adding methylglyoxal, the

Table IX. *Effect of glucose on glyoxalase activity of intact cells.*

Exp.	Time of exposure (min.)	Rate in N_2+5 % CO_2	Rate in O_2+5 % CO_2	Rate in O_2+5 % CO_2 +0·2 % glucose	
1	124	100	71·5	91	
2	128	100	69·5	79	
3	121	100	77	74	
4	121	100	55	92	
5*	120	100	29	84	Cells well
6	88	100	44	76	washed
Mean value		100		83	

* For details of this experiment see Table XII.

glyoxalase activity has fallen off less when glucose is present than in its absence. The relative effect is more pronounced when the cells are well washed. Glucose is however unable to prevent a lower rate being shown in oxygen than in nitrogen. The mean rate after exposure to glucose and oxygen is 83 % of that after exposure to nitrogen.

It is significant that although, as in the case of intact cells, oxygen effects a

considerable decrease in the glyoxalase activity of lysed cells to which glutathione[1] had been added, the addition of glucose to the latter does not appreciably diminish the magnitude of this effect. This result is recorded in Table X, where it will be observed there is no enhancement by glucose (within the limits of experimental error) of the glyoxalase activity of lysed cells in the presence of oxygen.

Table X. *Effect of glucose on glyoxalase activity of lysed cells (+ GSH).*

Exp.	Rate in $N_2+5\%\ CO_2$	Rate in $O_2+5\%\ CO_2$	Rate in $O_2+5\%\ CO_2$ $+0{\cdot}2\%$ glucose	Time of exposure before addition of methylglyoxal (min.)
1	100	69	71	85
2	100	45	46	128

In Table XI are shown experiments which demonstrate that the lowering in the glyoxalase activity of intact cells can be reversed by restoring them to nitrogen. Cells were shaken in oxygenated Ringer solution in presence of glucose for 1–2 hours, two manometric vessels being similarly filled and treated. At the

Table XI. *Effect of transferring to N_2 intact cells previously exposed to O_2 (in presence of glucose).*

Exp.	Rate in $O_2+5\%\ CO_2$	Rate in $N_2+5\%\ CO_2$
1	83	100
2	83	100
3	82	100
4*	84	100

* For details of this experiment see Table XII.

Table XII. *A typical experiment showing the effect of oxygen and glucose on the glyoxalase activity of intact red blood cells.*

In each vessel 0·015 cc. of concentrated cell suspension (well washed with saline, dry weight 4·87 mg.) in a total volume of 2·9 cc. Ringer solution. Vessels shaken at 37° for 120 minutes before adding to cell suspension 1·7 mg. methylglyoxal in 0·3 cc. saline-bicarbonate.

In vessel 4 the O_2+CO_2 previously present was replaced by N_2+CO_2 about 10 minutes before adding the methylglyoxal.

Vessel ...	1	2	3	4
Gas ...	N_2	O_2	O_2	$O_2 \rightarrow N_2$
% glucose ...	—	—	0·2	0·2
Time (mins.)		mm.^{3} CO_2 evolved		
5	24	8	18	32
15	76·5	21	61	82
25	129	38	107	136
35	180	53	149	187
45	230	67	190	238
55	278	81	232	289
Average rate (mm.^{3}/min.) 5–45 mins.	5·15	1·47	4·30	5·15
Ratio of rates	(100)	29	84	100

[1] No attempt has been made by us to use "stabilised" glutathione.

end of this period, the oxygen in one of the two vessels was replaced with nitrogen, and methylglyoxal was then added to the cells in both vessels. The cells showed a higher glyoxalase activity when nitrogen replaced oxygen.

The effect of oxygen in lowering glyoxalase activity is evidently reversible, at least in presence of glucose. The increase in rate is from 83 to 100.

The above experiments show that oxygen has a reversible effect on glyoxalase activity. Oxygen lowers the activity reversibly by 17–20 %, but can produce larger effects when the washed cells are shaken in absence of added glucose[1] for some time.

Discussion.

The work described indicates that the presence of glutathione is necessary for the activity of glyoxalase of red blood cells, confirming the work of Lohmann on tissue extracts. The results with lysed cells show that the red blood cell contains ample glutathione to account for its glyoxalase activity and that the disappearance of glyoxalase activity on completely lysing corpuscles is due to dilution of the cell contents. The thermolabile glyoxalase itself is probably fairly stable and unaffected by lysis of the corpuscle. The experiments recorded also show that methylglyoxal and glutathione combine reversibly. The following scheme therefore represents, we suggest, the course of transformation of methylglyoxal into lactic acid, catalysed by glyoxalase:

$$CH_3COCHO + HS.G \rightleftarrows CH_3COCHOH.SG \xrightarrow{H_2O} CH_3CHOHCOOH + GSH.$$

The exact nature of the union between glutathione and methylglyoxal has still to be decided upon, but that given seems to us to be most likely.

Lohmann [1932] has shown that oxidised glutathione does not act as a co-enzyme for glyoxalase. It follows then that the effect of oxygen in lowering glyoxalase activity is probably due to partial oxidation of glutathione, which results in a lowering of the co-enzyme concentration and hence of the activity of the enzyme system. The reversibility of the effect of oxygen makes it probable that the oxidised glutathione can be reduced again in the red cell, and thereby become active again as a co-enzyme.

The recent work of Hopkins and Elliott on liver [1931] has shown that oxidation and reduction of glutathione proceed in this tissue, and that the reduction is probably dependent on the presence of oxidisable substances. A dynamic equilibrium between oxidation and reduction is supposed to exist. This view of the relation between oxidation and reduction exactly fits our experiments on red blood cells, which may be interpreted by supposing that in the presence of oxygen glutathione is oxidised, but that in presence of glucose or residual metabolites the cell can reduce oxidised glutathione. A dynamic equilibrium is set up in oxygen at a level of reduced glutathione concentration which brings about a glyoxalase activity about 80–83 % of that in absence of oxygen. In the absence of added glucose, the oxidisable substances left in the red cells after washing are in time partially removed (either by oxidation or by diffusing out of the cells), and oxidised glutathione is less rapidly reduced in the cells. Hence the glyoxalase activity falls further. This explanation receives support from the more rapid fall in glyoxalase activity when the cells are more thoroughly washed with saline.

[1] It should be noted that gas changes due to glycolysis and respiration of red blood cells are too small to affect our measurements of glyoxalase activity.

As direct evidence that reduction and oxidation of glutathione take place in red blood cells the work of Meldrum [1932] on the red cells of the ox may be cited. This author found that on aerating concentrated suspensions of the cells at 37° their glutathione was slowly oxidised; in presence of glucose the reduced glutathione present in the cells was increased. Glucose had no effect on the glutathione content when the cells were lysed, which fits in with our experiments (Table X) on the glyoxalase activity of lysed cells.

In view of this work, it seems very probable indeed that the glyoxalase activity of the red cell under varying conditions is an indicator of the concentration of reduced glutathione in the cell.

The fact that the glyoxalase activity of the red blood cell has different levels under anaerobic and aerobic conditions has, possibly, other bearings. There is an obvious similarity to the different levels of glycolytic activity shown by tissues under anaerobic and aerobic conditions [Warburg, 1926]. The glycolytic phenomenon is a general one, whereas the effects of oxygen on glyoxalase activity have not been described before, so far as we are aware, and experiments have so far been made only on one type of cell. Whether the glyoxalase activity of tissues in general will be lowered by oxygen in a reversible manner remains to be investigated.

It seems, however, legitimate to suggest that a partial explanation of the Pasteur reaction may be found in the effect of oxygen in lowering the concentration of reduced glutathione in cells, and thereby lowering their glyoxalase activity. Let us suppose (1) that the final stage in glycolysis is the transformation of methylglyoxal into lactic acid, and (2) that this process is, under the conditions obtaining in cells, slow enough to exert an influence on the rate of the sequence of processes that take place in glycolysis. On these suppositions a qualitative explanation of the Pasteur reaction follows.

The theory receives some support from the recent findings of Bumm and Appel [1932] that in the presence of added glutathione the effect of oxygen on the glycolysis of tumour cells disappears. In terms of the present theory, the glutathione concentration of the cells is raised, and hence also the glyoxalase activity, which, we are suggesting, influences the rate of glycolysis. It is significant for the general problem of metabolism of glucose that, according to Quastel and Wheatley [1932], glutathione also raises the aerobic fermentation of glucose by yeast to the anaerobic level.

Summary.

1. Human red blood corpuscles show a high glyoxalase activity. This activity almost entirely disappears on lysis of the cells with distilled water.

2. The activity of the glyoxalase of lysed cells is restored to about the original level of the intact cells by the addition of glutathione at concentrations of the same order as that present in the intact cell.

3. Evidence is presented which shows that methylglyoxal and glutathione combine reversibly. The evidence is based on three lines of investigation.

(*a*) Manometric, indicating the formation of a compound more acid than glutathione itself.

(*b*) Iodimetric, indicating the union of —SH with the methylglyoxal.

(*c*) By the effect of glutathione on the glyoxalase activity of the red blood cell.

It is suggested that the transformation of methylglyoxal, catalysed by the enzyme, takes the following course:

$$CH_3COCHO + G.SH \rightleftharpoons CH_3COCHOH.SG \xrightarrow{H_2O} CH_3CHOHCOOH + G.SH.$$

4. Oxygen diminishes the glyoxalase activity of both intact and lysed red blood cells.

5. In the presence of glucose, the inhibitory effect of oxygen on the glyoxalase activity of the intact red blood cell is decreased; the behaviour of the lysed blood cell on the other hand is unaffected by glucose.

6. The effect of oxygen on the glyoxalase activity of the red blood cell is reversible. Transference of oxygenated cells to nitrogen restores the anaerobic level of glyoxalase activity.

7. The effects of oxygen and glucose on the glyoxalase activity of the red blood cell may be interpreted in terms of an equilibrium between oxidised and reduced glutathione in this cell.

8. The parallelism between the effects of oxygen on glyoxalase activity and glycolytic action is discussed in relation to the Pasteur reaction.

We wish to thank the Medical Research Council for a grant towards the equipment of this laboratory and for a whole time grant to one of us (M. J.). We wish also to thank Mr A. H. M. Wheatley for assistance in carrying out the lactic acid determinations.

REFERENCES.

Bumm and Appel (1932). *Z. physiol. Chem.* **210**, 79.
Friedemann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.
Hopkins and Elliott (1931). *Proc. Roy. Soc. Lond.* B **109**, 58.
Jowett (1932). *Biochem. J.* **26**, 2108.
King, Baumgartner and Page (1930). *Biochem. Z.* **217**, 389.
Kühnau (1931). *Biochem. Z.* **243**, 14.
Lohmann (1932). *Biochem. Z.* **254**, 332.
Meldrum (1932). *Biochem. J.* **26**, 817.
Quastel and Wheatley (1932). *Biochem. J.* **26**, 2169.
Warburg (1926). Über den Stoffwechsel der Tumoren. (Berlin.)
—— and Christian (1931). *Biochem. Z.* **242**, 206.
—— Posener and Negelein (1924). *Biochem. Z.* **152**, 309.

LXXI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXVIII. HELMINTHOSPORIN AND HYDROXY*ISO*-HELMINTHOSPORIN, METABOLIC PRODUCTS OF THE PLANT PATHOGEN *HELMINTHOSPORIUM GRAMINEUM* RABENHORST.

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(*Received February 28th, 1933.*)

During the routine examination and sub-culturing of our stock collection of fungi, it was noticed that cultures of *Helminthosporium gramineum* Rabenhorst (Catalogue No. Ag. 106), both on beer wort agar and Czapek-Dox agar slopes showed a particularly vivid red coloration of the medium. This fungus is a plant pathogen and is the causative agent of the well known "leaf stripe" disease of barley. The red coloration had been apparent in the stock cultures for a long time without a great deal of significance being attached to it. On this occasion, however, microscopical examination of the cultures showed that below the aerial mycelium, which was floccose and cottony in appearance, the basal and submerged mycelium showed large areas with what appeared to be cell contents of a deep red colour. In addition a good deal of the mycelium in these lower strata appeared to be partially covered with dark-red needle-shaped crystals.

Cultures of *H. gramineum* (Ag. 106) were therefore made on aqueous liquid media, in the one case on 350 cc. Czapek-Dox solution containing 5 % glucose, and in the other case on 350 cc. beer wort. On Czapek-Dox solution after 42 days' incubation a strong felt of mycelium (dry weight 2·65 g.) had developed, the surface of which was grey in colour and the reverse a dark red. On liquid beer wort a heavier growth of mycelium (dry weight 7·65 g.) was obtained, the aerial part of which was quite white and cottony, while the basal mycelium was again a dark red. No spores were produced in either case. A microscopic examination of the mycelium revealed in both cases well-formed very dark-red needle-shaped crystals, which dissolved in a drop of caustic soda to give a deep-violet coloured solution. A photomicrograph of *H. gramineum* (Ag. 106) grown on Czapek-Dox glucose solution and showing the typical crystalline material is given in Fig. 1. Examination showed that this material is a mixture and we have been able to separate two pure substances from it by comparatively simple methods (see p. 509).

The main constituent is the more sparingly soluble and we propose to term it *helminthosporin*. This substance has the formula $C_{15}H_{10}O_5$, and it is thus isomeric with emodin; it was indeed at once apparent that the pigment displayed the character of a polyhydroxyquinone.

The constitution of the substance was determined in the following manner.

On distillation over zinc dust in a stream of hydrogen, 2-methylanthracene was produced. The *triacetate* of helminthosporin was found to be a yellow, neutral substance. It followed that helminthosporin is a trihydroxy-2-methylanthraquinone and dyeing trials showed that it is not a derivative of alizarin.

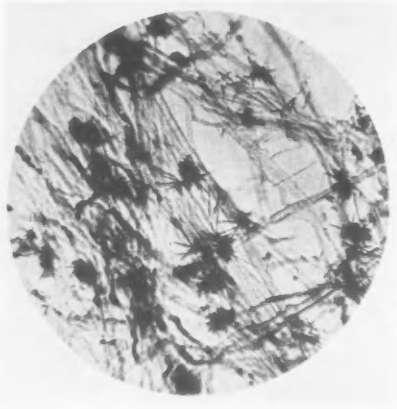
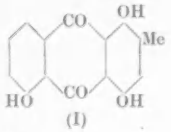


Fig. 1.

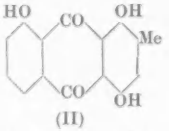
In order to determine whether all three hydroxyl groups are situated in the aromatic nuclei or whether one of them occurs as a methylol group (as in aloemodin), triacetylhelminthosporin was oxidised with chromic acid in acetic acid—acetic anhydride solution. The product was triacetylhelminthosporic acid and hence the three hydroxyls are nuclear. On hydrolysis helminthosporic acid, $C_{15}H_8O_7$, was obtained, the decarboxylation of which proved very difficult. Ultimately it was accomplished by heating the acid with quinoline and copper chromite, and the product proved to be 1:4:5-trihydroxyanthraquinone.

This substance has been scantily described (D.R.P., 161,026; 162,035; 162,041) but as all the recorded methods of preparation are quite unsatisfactory and no M.P. is quoted, or derivative described, it may be said to be hitherto unknown. We obtained it by the action of aqueous calcium hydroxide on 1:4:5-trichloroanthraquinone at 230° and found the product (M.P. 269–270°) to be identical with that from helminthosporic acid.

Helminthosporin was thus recognised as one of four possible 1:4:5-trihydroxy-β-methylanthraquinones (I, II, III, IV).



(I)



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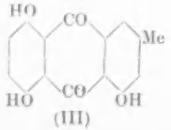

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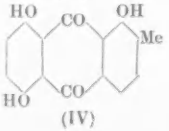


(IV)

A definite decision between these was afforded by the results of oxidation with concentrated nitric acid. This gave in addition to oxalic acid and a small amount of a trinitrohelminthosporin, a considerable quantity of nitrococcusic acid (2:4:6-trinitro-5-hydroxy-3-methylbenzoic acid) whose identity was established by direct comparison with an authentic specimen prepared by nitration of 5-hydroxy-*m*-toluic acid and by conversion into trinitro-*m*-cresol. On this basis helminthosporin is 2-*methyl*-4 : 5 : 8-*trihydroxyanthraquinone* (III).

The more soluble constituent of the crude product has the empirical composition, $C_{15}H_{10}O_6$, and its general character closely resembles that of helminthosporin. The additional oxygen atom is present as hydroxyl, the substance furnishing a tetra-acetate.

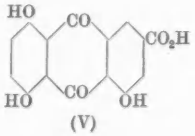
At first we thought the substance to be a hydroxyhelminthosporin but this is now considered improbable for the following reasons.

(1) The substance $C_{15}H_{10}O_6$ is not an alizarin derivative; its dyeing properties are similar to those of helminthosporic acid.

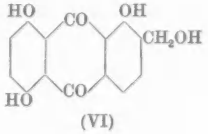
(2) It is improbable that it can be 1:4:5:8-tetrahydroxy-2-methylanthraquinone because this constitution must be reserved for a different substance which can be obtained (although not as yet in an analytically pure condition) by the action of manganese dioxide on helminthosporin in sulphuric acid solution.

(3) It is improbable that it can be ω-hydroxyhelminthosporin (1:4:5-trihydroxy-7-hydroxymethylanthraquinone) because its tetra-acetate does not afford triacetylhelminthosporic acid on oxidation under the conditions that succeed with triacetylhelminthosporin. Yet all experience shows that $.CH_2OAc$ is more readily oxidisable than $.CH_3$, a comparison in point being the ready oxidation of triacetylaloemodin as contrasted with the much more difficult attack of diacetylchrysophanic acid.

These considerations exclude the view that the substance is hydroxyhelminthosporin but it is doubtless very closely related to helminthosporin and we propose for it the name *hydroxyisohelminthosporin*. On oxidation with manganese dioxide and sulphuric acid it does not yield an alizarin derivative and hence it seems unlikely that there are more than three nuclear hydroxyls. Its similarity to helminthosporic acid (V) is expressed in the provisional formula VI which also allows of an explanation of the resistance offered by the side-chain towards oxidising agents.



(V)



(VI)

Naturally there are other similar possibilities and the investigation of hydroxy-*iso*helminthosporin is not yet completed.

Helminthosporin has been synthesised and this confirmation of our deductions will be published in another place.

The investigation of the pigments produced by a large number of other species of *Helminthosporium* has been commenced and has given interesting results which it is hoped will be communicated to the Society in due course.

Experimental.

Effect of various factors on pigment production.

1. *Temperature of incubation.* Cultures of *H. gramineum* (Ag. 106) were made on slopes of beer wort agar and Czapek-Dox glucose agar, and incubated at room temperature (18–20°), 24°, 27°, 30°, 32°, 34° and 37°. The cultures were examined at intervals up to 1 month. No growth was obtained on either medium at 34° or 37°. At the lower temperatures, especially at room temperature, a profuse mycelial growth was obtained on both media, although with less of the pigment than was produced at the higher temperatures. Pigment production appeared to be greater on Czapek-Dox agar than on beer wort agar. Maximum production of pigment appeared to be obtained at 27–30° and this temperature range was therefore subsequently used as the standard incubation temperature.

2. *Sources of nitrogen.* A quantity of the usual Czapek-Dox agar without a source of nitrogen was made up, having the following composition: glucose, 50 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4, 7H_2O$, 0·5 g.; $FeSO_4, 7H_2O$, 0·01 g.; agar-agar, 20 g.; water 1000 cc. To this was then added sufficient of the following sources of nitrogen to give a nitrogen concentration equivalent in each case to *M*/40 N: sodium nitrate, potassium nitrate, calcium nitrate, ammonium nitrate, ammonium chloride, ammonium sulphate, ammonium tartrate, sodium ammonium phosphate, urea, peptone, asparagine and gelatin. The nitrogen content of peptone and gelatin was assumed to be 16 %. Cultures of *H. gramineum* (Ag. 106) were then made on each of these media and incubated at 27°. It was found that ammonium nitrate, chloride, sulphate and tartrate gave unsatisfactory growth and little if any pigment production. The largest amount of pigment was produced in the tubes containing sodium, potassium and calcium nitrates, and smaller amounts with microcosmic salt, asparagine, urea, peptone and gelatin.

3. *Sources of carbon.* Czapek-Dox agar, without a source of carbon other than agar, and of the following composition was made up: $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4, 7H_2O$, 0·5 g.; $FeSO_4, 7H_2O$, 0·01 g.; agar-agar, 20 g.; water, 1000 cc. To portions of this were then added 5 % of one of each of the following sources of carbon: sucrose, maltose, glucose, fructose, mannose, galactose, xylose, arabinose, mannitol, glycerol and soluble starch. Slopes of these media were then sown with *H. gramineum* (Ag. 106) and incubated at 27°. After 39 days' incubation a strong mycelial felt was obtained in all cases, but no sporulation was obtained in any case. No red colouring matter could be detected with arabinose or glycerol, only traces with galactose and mannitol, a small amount with xylose, while considerable and comparable amounts were obtained with sucrose, maltose, glucose, fructose, mannose and soluble starch.

4. p_H *of medium.* A quantity of Czapek-Dox medium having the following composition was made up: glucose, 50 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4, 7H_2O$, 0·5 g.; $FeSO_4, 7H_2O$, 0·01 g.; water, 1000 cc. 350 cc. quantities of this medium were measured into a series of 1-litre conical flasks and sterilised. After sterilisation the appropriate amount, previously determined, of sterile solutions of *N* HCl or *N* NaOH were added to separate flasks to bring the p_H values to 2·0, 3·0, 4·0, 5·0, 6·0, 7·0, 8·0, 8·5 and 9·0 respectively. One flask at each p_H was then sown with *H. gramineum* (Ag. 106) and incubated at 30° for 1 month. The mycelium was filtered, washed, dried and weighed. The dried mycelium was then powdered, exhaustively extracted in a Soxhlet apparatus with ethyl ether, and the weight of the crude ether extract determined. The results obtained are given in Table I.

Table I.

p_H	Dry weight of mycelium, g.	Weight of crude ether extract, g.
2·0	Nil	Nil
3·0	0·54	0·08
4·0	1·06	0·14
5·0	1·86	0·29
6·0	2·59	0·80
7·0	3·06	0·91
8·0	3·06	1·02
8·5	3·07	0·63
9·0	2·76	0·48

Since ethyl ether completely extracts the pigments it is obvious that pigment production on this medium is at a maximum at p_H 8·0, although the yield of pigment is considerably smaller than the weight of the crude ether extract, as the latter contains considerable amounts of fat.

5. *Species or strains of* Helminthosporium *used.* The strain of *H. gramineum* (Ag. 106) was originally isolated in Mr F. T. Brooks's Laboratory, School of Botany, Cambridge, in 1925. It was isolated from barley infected with "leaf stripe" disease, and it has been maintained in stock culture either on beer wort agar or Czapek-Dox agar ever since. It is now a non-sporing form and has been so for the greater part of the time since its first isolation. The red coloration has become gradually more intense through successive sub-cultivation. In view of this fact, and also in view of the biochemical interest of the pigments produced, other strains were examined. One of these (Cat. No. Ag. 98) was the type strain of *H. gramineum* received in 1925 from the Centraalbureau voor Schimmelcultures, Baarn, Holland. This strain was also a non-sporing form. In order to remove all doubt a sample of infected barley leaf showing typical "leaf stripe" disease was obtained through the courtesy of Mr F. T. Brooks in May 1932. This material showed characteristic mycelium bearing typical conidia. A single spore isolation was made and a culture transferred to Czapek-Dox agar. The first culture (Cat. No. HgO) showed distinct signs of orange coloration along the edges of the growth. The second sub-culture on Czapek-Dox agar showed a decidedly more intense red coloration, which was fairly general on the reverse of the mycelium, and a microscopic examination at the edges showed strands of the mycelium containing red pigment as a cell inclusion. The production of red pigment became more marked with successive sub-cultivation and finally a small amount of crude red pigment, identical in nature with that obtained with culture No. Ag. 106, was obtained from this strain (Cat. No. HgO) and also from the type strain Ag. 98.

Preparation of crude pigment.

35 litres of Czapek-Dox medium of the following composition were made up: glucose, 50·0 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4,7H_2O$, 0·5 g.; $FeSO_4,7H_2O$, 0·01 g.; distilled water, 1000 cc. 350 cc. quantities of this medium were distributed in each of 100 1-litre conical flasks and sterilised. A measured quantity, previously determined, of sterile *N* sodium hydroxide solution was added to each flask to bring the p_H of the medium to 8·0. Each flask was then sown with a suspension of *H. gramineum* (Ag. 106) and incubated at 30° for 6 weeks. At the end of this time the mycelium, which showed the typical deep red reverse, was filtered off, washed carefully with cold water, pressed and dried in a vacuum-oven. The dried mycelium in three experiments

weighed 247, 272 and 285 g. respectively. It was ground to a fine powder in a coffee mill and exhaustively extracted with ethyl ether, acetone or chloroform in a large Soxhlet apparatus. Of these solvents chloroform was found to be the most efficacious. After some time a dark red crystalline solid separated from the solvent and was filtered off each day. The total yields of crude extract in each of the above experiments were 100·7, 109·6 and 117·8 g., while the total yields of crude pigment were 74·6, 76·6 and 82·0 g. respectively. The portion of the crude extract other than pigment consisted of a yellowish oil. The yields of crude pigment obtained thus corresponded to 30·2, 28·2 and 28·8 % of the dry weight of the mycelium and to 4·26, 4·38 and 4·69 % of the glucose in the medium.

Properties and composition of crude pigment.

The crude pigment consists of a dark red crystalline material which is obviously a mixture. Two types of crystals appear in the extract, and the crude product melts indefinitely from about 160° upwards. It may be practically completely sublimed in a high vacuum at 140–150°. The main constituents of the crude pigment are *helminthosporin*, $C_{15}H_{10}O_5$, and *hydroxyisohelminthosporin*, $C_{15}H_{10}O_6$, approximately two to three times as much of the former as of the latter. The solubilities of an average sample of the crude pigment in various solvents are as follows: it is very sparingly soluble in hot water, moderately soluble in boiling methyl alcohol, fairly readily soluble in boiling acetone, ethyl alcohol, benzene or ethyl acetate, readily soluble in boiling glacial acetic acid or hot chloroform.

Isolation and properties of helminthosporin (III).

In order to obtain a sample of pure helminthosporin for analysis, the following procedure was adopted: 1·0 g. of the crude pigment was dissolved in 45 cc. hot glacial acetic acid. The first crop of crystals consisting of 0·65 g. of dark red pointed needles, M.P. 184–190°, was recrystallised twice from ethyl acetate (100 and 60 cc. respectively), yielding 0·14 g. of maroon needles, M.P. 205–218°, which were much darker in colour than the parent first crop from glacial acetic acid. A final crystallisation from chloroform (about 10 cc.) yielded 0·05 g. of very dark maroon, flat, tapering needles, M.P. 225–226° (sharp without decomposition). (Found: C, 66·7, 66·6; H, 3·8, 3·7 %; mol. wt., cryoscopic in camphor, 302, 313. $C_{15}H_{10}O_5$ requires C, 66·6; H, 3·7 %; mol. wt. 270.) (Microanalyses by Schoeller.)

This sample of pure helminthosporin, M.P. 225–226°, dissolved sparingly in alcohol, acetic acid, ether and acetone to give orange-red solutions with a green fluorescence; it gave the following colour reactions.

With concentrated sulphuric acid in the cold a beautiful and intense cherry-red colour is obtained, which is purple in thin layers and becomes very much bluer in shade on warming gently after the addition of a few crystals of sodium nitrite. If the original cherry-red solution is heated and then poured into water, a golden-orange solution is obtained, the colour changing to violet on the addition of excess of ammonia. The weak red fluorescence of the solution in sulphuric acid becomes much stronger on the addition of boric acid; at the same time the solution becomes bluer in tone.

With concentrated nitric acid, there is no coloration in the cold, but on warming a cherry-red colour develops, becoming brownish-red on further heating. If this solution is then diluted with water and made alkaline by the addition of sodium hydroxide solution a pale green colour is obtained.

Aqueous sodium hydroxide solution gives a deep bluish-red colour, somewhat similar to that given with concentrated H_2SO_4.

Aqueous barium hydroxide solution gives no colour owing to the sparing solubility of the barium salt.

An alcoholic solution of helminthosporin, which was orange in colour with a strong green fluorescence, was used, after dilution with water, for the following tests.

(*a*) The addition of aqueous solutions of ammonia, $NaHCO_3$, Na_2CO_3 or borax gives a beautiful violet solution which is immediately decolorised on the addition of aqueous sodium hypochlorite solution.

(*b*) Aqueous nickel sulphate solution gives a rose-pink colour with a green fluorescence. On warming, the colour becomes more intense but soon fades on standing, a brown flocculent precipitate being formed.

(*c*) Aqueous ferric chloride solution gives only a pale greenish-brown coloration.

(*d*) If the alcoholic solution is shaken with a little pyridine and then poured into water, an intense pink coloration with a slight brown tinge is obtained.

(*e*) Aqueous potassium or ammonium alum solution gives no coloration in the cold, but on warming a beautiful eosin-pink coloration develops with an orange fluorescence.

(*f*) Aqueous magnesium chloride solution gives a stable, intense, slightly bluish-pink coloration in the cold.

2-Methylanthracene from helminthosporin.

An intimate mixture of helminthosporin (1 g.) and zinc dust (20 g.) was subjected to distillation in a combustion tube at a dull red heat in a slow current of hydrogen. 2-Methylanthracene was deposited in the cooler part of the tube as colourless leaflets with a fine green fluorescence. After recrystallisation from alcohol the substance had M.P. 198° and exhibited all the properties recorded of 2-methylanthracene. For further confirmation the related quinone was prepared. The product from the zinc dust distillation of helminthosporin (1 mol.) was dissolved in hot glacial acetic acid, and a solution of chromic anhydride (2 mols.) in glacial acetic acid was added gradually, the solution being kept gently boiling. After all the oxidising agent had been added, the mixture was diluted with water and allowed to cool when small yellowish needles separated, M.P. 172°. The identity of this product with 2-methylanthraquinone (M.P. 173–174°) was confirmed by the fact that a mixture with an authentic specimen of that substance had M.P. 173°.

Triacetylhelminthosporin.

Helminthosporin (3 g.) was boiled for a few minutes with an excess of acetic anhydride and a drop of concentrated sulphuric acid. The clear yellow solution was then poured into ice-water, and after standing for several hours the solid precipitate was collected and recrystallised several times from a mixture of acetic anhydride (1 vol.) and acetic acid (3 vols.). Triacetylhelminthosporin was thus obtained as a mass of pale yellow needles, M.P. 223–224°. (Found: C, 63·6, 63·6; H, 4·1, 4·3 %; $C_{15}H_7(OCOCH_3)_3O_2$ requires C, 63·6; H, 4·0 %.)

Oxidation of triacetylhelminthosporin to triacetylhelminthosporic acid.

Triacetylhelminthosporin (3 g.) was dissolved in a mixture of glacial acetic acid (60 cc.) and acetic anhydride (60 cc.) at 55°, and a solution of chromic anhydride (6 g.) in water (6 cc.) and glacial acetic acid (30 cc.) was added in

small portions with shaking during half an hour. Considerable heat was evolved in the reaction and addition of the chromic anhydride was regulated so as to keep the temperature between 65 and 70° (the reaction having been found by trial to proceed most smoothly under these conditions). After all the chromic acid had been added the mixture was heated for 15 minutes on the steam-bath and the clear green solution mixed with hot water (500 cc.). On cooling and allowing to stand the product separated in small, pale yellow crystals (2 g.). A small quantity of unchanged triacetylhelminthosporin was removed by dissolving the crude substance in cold dilute sodium carbonate, filtering and acidifying the filtrate. The precipitate was dissolved in a small amount of acetic anhydride, adding a drop of concentrated sulphuric acid and boiling to ensure complete acetylation, and allowed to crystallise. Triacetylhelminthosporic acid crystallises from a mixture of acetic anhydride and acetic acid in lemon-yellow needles. After several recrystallisations it had M.P. 235–236°. (Found: C, 58·8; H, 3·5 %. $C_{21}H_{14}O_{10}$ requires C, 59·2; H, 3·3 %.)

Helminthosporic acid (V).

Triacetylhelminthosporic acid (1·5 g.) was hydrolysed by heating on the water-bath for 2 hours with aqueous sodium hydroxide (40 cc. of 10 %). The deep violet-coloured solution was acidified with hydrochloric acid, the mixture boiled for a few minutes to coagulate the gelatinous precipitate of helminthosporic acid and filtered. The solid thus obtained was recrystallised from pyridine from which it separated as small red-brown needles (0·85 g.) containing solvent of crystallisation. After two recrystallisations it had M.P. 359–360° undergoing partial sublimation above 330°, the sublimed material having the form of stout dark red needles. (Found: C, 62·0; H, 3·3; N, 2·2 %. $C_{15}H_8O_7$, 0·5 C_5H_5N requires C, 61·9; H, 3·1; N, 2·1 %.)

Helminthosporic acid is practically insoluble in ether and alcohol and is sparingly soluble in pyridine and nitrobenzene. Solutions in alkalis are violet in colour; the solution in concentrated sulphuric acid is cherry-red with a weak red fluorescence, which is intensified on addition of boric acid. On heating with acetic anhydride containing a drop of sulphuric acid for a few minutes, triacetylhelminthosporic acid, M.P. 235–236°, is obtained.

Helminthosporic acid is a weak mordant dye giving reddish-violet shades on cotton mordanted with aluminium; it is, however, much more effective than helminthosporin, the dyeing properties of the latter being very poorly developed indeed.

Ethyl helminthosporate. This ester can be prepared by heating helminthosporic acid with alcoholic sulphuric acid, but, as the reaction is very slow owing to the low solubility of the acid, the following method was adopted.

Triacetylhelminthosporic acid (1·6 g.) was boiled for an hour with absolute alcohol (50 cc.) containing concentrated sulphuric acid (5 cc.). The acetyl compound passed into a yellow solution which rapidly changed through orange to red and the ethyl ester of helminthosporic acid separated slowly as a mass of fluffy red needles. These were collected, washed with alcohol and recrystallised from a mixture of chloroform and alcohol. The yield was almost theoretical, and the pure ester had M.P. 194–195° after sintering slightly at 191°. (Found: C, 62·6; H, 3·7 %. $C_{17}H_{12}O_7$ requires C, 62·2; H, 3·7 %.)

Attempts to convert this ester into the corresponding hydrazide by heating with hydrazine hydrate were unsuccessful, the product being a brown substance which did not melt at 400°, was insoluble in acids and soluble with difficulty in

sodium hydroxide solution. This material contained nitrogen but even after extraction with alcohol in a Soxhlet apparatus it did not give concordant analyses.

Triacetyl derivative of ethyl helminthosporate. Ethyl helminthosporate was acetylated in the usual manner with acetic anhydride containing sulphuric acid. The product crystallised from acetic acid in fine orange-yellow needles, M.P. 205–206°. (Found: C, 60·0; H, 4·0 %. $C_{23}H_{18}O_{10}$ requires C, 60·8; H, 4·0 %.)

Decarboxylation of helminthosporic acid. Formation of 1:4:5-trihydroxyanthraquinone.

Helminthosporic acid is an exceedingly stable compound—it even appears to be capable of sublimation—and for a time all our efforts to decarboxylate it were fruitless. Thus the usual methods (*e.g.* heating under pressure with water or pyridine, or heating with glycerol) yield either unchanged starting material or, in the case of the pyridine method, complex substances of unknown constitution. Similarly an attempt to replace the carboxyl by an amino-group by the action of hydrazoic acid in sulphuric acid solution (compare Schmidt, D.R.P. 500,435) was unsuccessful.

Ultimately the end was achieved by taking advantage of the catalytic effect on the decarboxylation of acids produced by mixtures of quinoline and copper chromite [Kinney and Langlois, 1931].

Helminthosporic acid (2·4 g.) was heated with pure quinoline (15 cc.) and copper chromite (0·3 g.) (prepared according to the method of Adkins and Folkers [1931]) in a glass tube immersed in a paraffin-bath. Slow evolution of gas occurred when the temperature reached 200° and the bath was maintained at 220–230° during 2 hours. After cooling, the dark red contents of the tube were treated with excess of dilute hydrochloric acid to remove quinoline, the mixture boiled and filtered. The solid thus obtained was extracted with cold dilute sodium carbonate solution which removed a quantity of unchanged helminthosporic acid (0·35 g.) leaving a dark brown residue (1·4 g.) containing copper chromite. On recrystallising from pyridine, the product was obtained as small, dark red-brown needles with a rather indefinite M.P., 260–265°. Further purification was effected by conversion into the acetyl derivative (below), the free hydroxy-compound being regenerated from the pure acetate by warming on the steam-bath with aqueous potassium hydroxide (15 %) during 90 minutes. After several recrystallisations from pyridine this material was obtained as deep red needles, M.P. 268–269°, accompanied by slight sublimation. The analytical results show it to be a trihydroxyanthraquinone. (Found: C, 65·6; H, 3·3 %. $C_{14}H_8O_5$ requires C, 65·6; H, 3·1 %.)

Acetyl derivative. This was prepared by boiling the trihydroxyanthraquinone with acetic anhydride containing a drop of concentrated sulphuric acid in the usual way; it crystallised from acetic acid in pale yellow needles, M.P. 224–225°, after slight sintering.

1:4:5-Trihydroxyanthraquinone. 1:4:5-Trichloroanthraquinone[1] (2 g.) [compare Goldberg, 1931] was mixed into a paste with slaked lime (8 g.), copper bronze (0·2 g.), and water (25 cc.), and the mixture heated in a sealed tube at 230° for 20 hours. After cooling, the violet-coloured paste was washed out of the tube with excess of dilute hydrochloric acid which produced a reddish-brown precipitate of the trihydroxyanthraquinone. The mixture was boiled for a few minutes to coagulate the precipitate and filtered, the residue being crystallised

[1] The material employed was actually prepared by the late Dr A. W. Fyfe at Dalton Works, Huddersfield, in 1920. The method used was similar to that of Goldberg.

from pyridine when it formed red-brown needles (0·9 g.) of indefinite M.P., 252–258°. It was purified by conversion into the acetyl derivative (see below) and hydrolysis of the purified acetate by means of warm aqueous potassium hydroxide. The precipitate obtained on acidifying the red-violet alkaline solution was recrystallised from pyridine when it formed deep red needles, M.P. 270–271°, accompanied by slight sublimation. (Found: C, 65·8; H, 3·3 %. $C_{14}H_8O_5$ requires C, 65·6; H, 3·1 %.)

1:4:5-Triacetoxyanthraquinone. Acetylation of the above substance with acetic anhydride and sulphuric acid gave a product crystallising from glacial acetic acid in pale yellow needles, M.P. 225°, after slight sintering. (Found: C, 62·7; H, 3·8 %. $C_{20}H_{14}O_8$ requires C, 62·8; H, 3·7 %.)

Comparison of 1:4:5-trihydroxyanthraquinone with the trihydroxyanthraquinone from helminthosporic acid. (*a*) A mixture of the two compounds melted at 270°, while a mixture of their acetates melted at 224–225°.

(*b*) Both substances were insoluble in cold dilute sodium carbonate solution but dissolved in sodium hydroxide to identical reddish-violet solutions which decolorised slowly on standing in the air.

(*c*) Both dissolved in concentrated sulphuric acid to cherry-red solutions which were violet in thin layers and had a weak red fluorescence which became intense on addition of boric acid, the solutions becoming at the same time slightly bluer in tone.

There appears to be no doubt as to the identity of the two substances despite the slight discrepancy in melting-point. This latter fact may possibly indicate the presence in the helminthosporin used of small quantities of some closely related substance which has not yet been isolated.

Oxidation of helminthosporin with nitric acid.

Helminthosporin (3 g.) was warmed on the water-bath with nitric acid (30 cc., sp.gr., 1·42) when a violent reaction set in with copious evolution of brown fumes. After the reaction subsided the clear solution was heated on a boiling water-bath until evolution of brown fumes had practically ceased (about 1 hour), then concentrated to a small bulk and poured into water, when a small quantity of an orange-yellow solid was precipitated. The mixture was filtered.

Residue. As the amount obtained was very small this substance has not yet been thoroughly investigated. It crystallised from alcohol in deep yellow needles which on heating sintered at 220° and melted with decomposition at 225–227°. The analytical values obtained indicate that it is probably a trinitrohelminthosporin. (Found: N, 10·7 %. $C_{15}H_7O_{11}N_3$ requires N, 10·4 %.)

Filtrate. The filtrate was extracted several times with ether, the combined yellow extracts dried over sodium sulphate and the solvent removed, leaving a yellow crystalline residue. The aqueous layer, on evaporation to dryness, yielded oxalic acid (0·7 g.).

The ether extractive was thrice recrystallised from water when it formed a mass of magnificent, pale yellow rhombic plates (0·4 g.). This substance, though nearly colourless in the solid state, dissolves in water and many organic solvents to a bright yellow solution and is readily soluble in cold dilute sodium carbonate solution. The crystalline material contains water of crystallisation which it loses at 105°. On heating fairly rapidly it melts and decomposes at 199–200°, and explodes violently if suddenly heated to higher temperatures. (Found in material dried in the air: C, 31·8; H, 2·4; N, 12·9, 13·0; loss at 105°, 6·7 %. $C_8H_5O_9N_3, H_2O$ requires C, 31·5; H, 2·3; N, 13·7; H_2O, 5·9 %.) The analytical

values correspond with a trinitrohydroxymethylbenzoic acid and the properties of the substance agree with those of nitrococcusic acid (2:4:6-trinitro-5-hydroxy-3-methylbenzoic acid). A specimen of this latter acid was prepared according to the method of Kostanecki and Niementowski [1885]. The synthetic acid was colourless but proved identical with the acid from helminthosporin in every other respect—crystalline form, properties, M.P. and mixed M.P.

As an additional proof that the acid from helminthosporin is indeed nitrococcusic acid it was decarboxylated by heating with water in a sealed tube at 180° during 5 hours. On cooling, the product separated as a mass of long yellow needles. On recrystallisation from 40 % alcohol, long needles of trinitro-*m*-cresol, M.P. 109°, were obtained. The mixed M.P. with a sample of authentic trinitro-*m*-cresol was 109°.

Separation of helminthosporin and hydroxyisohelminthosporin from the crude material obtained by solvent extraction of the dried mould.

The various crops of crude pigment obtained in the manner indicated differed considerably in appearance, the earlier crops being bright red in colour while the later ones were brownish-red and also contained small amounts of sticky material. From the earlier crops it was possible to isolate the two distinct pigments helminthosporin (M.P. 225–226°) and hydroxy*iso*helminthosporin (M.P. 237–238°) which appeared to be present in the ratio of about 3:1, while the later crops consisted largely of helminthosporin mixed with various impurities (including some colourless material) which were not isolated.

The crude substance was extracted in a Soxhlet apparatus with 96 % alcohol when the extract was at first deep orange-red in colour. As soon as the liquid siphoning over began to lighten in colour the receiver was removed. On cooling, hydroxy*iso*helminthosporin separated in long, flat, red prisms and was purified by recrystallisation from alcohol.

The residue in the thimble was now further extracted with alcohol for a short time, giving a middle fraction, and then a mixture of alcohol and chloroform was substituted. The contents of the thimble now extracted continuously and the extracts on cooling deposited maroon-coloured crystals with a bronze lustre, M.P. about 200°. From this material by repeated crystallisation or by conversion into the acetyl derivative and purification of the latter, helminthosporin, M.P. 225–226°, was obtained. It crystallises from chloroform or pyridine in deep maroon-coloured crystals with a bronze lustre.

The low melting-point of the material obtained in the chloroform-alcohol extraction is rather surprising since no compound other than helminthosporin could be isolated from it in a pure condition. It is possible that the M.P. of helminthosporin is depressed considerably by the presence in the crude substance of a small amount of some closely related substance.

Hydroxyisohelminthosporin.

Hydroxy*iso*helminthosporin crystallises from alcohol in long thin rectangular plates of a bright red colour, M.P. 238°. The crystals contain solvent of crystallisation which is lost on heating. (Found in dried material: C, 63·4; H, 3·6 %. $C_{15}H_{10}O_6$ requires C, 62·9; H, 3·5 %.)

It is more soluble in most solvents than helminthosporin, its solutions resembling those of the latter in colour, and dissolves in cold dilute sodium carbonate solution or caustic alkali to a red-violet solution which is redder in tone than alkali solutions of helminthosporin. Solutions in concentrated

sulphuric acid differ widely from those of helminthosporin and 1:4:5-trihydroxyanthraquinone, being red-violet by transmitted light and intense blue-violet by reflected light. There appears to be little or no fluorescence, but the addition of boric acid produces a feeble red fluorescence.

The blue-purple colour of the solution in sulphuric acid does not change in shade on warming gently after the addition of a few crystals of sodium nitrite. When the original solution in sulphuric acid is heated and then poured into water, a red-brown precipitate is produced, which dissolves on the addition of excess of ammonia to a cherry-red solution.

With concentrated nitric acid there is no coloration in the cold, but on warming a pale cherry colour develops, becoming brownish on further heating. If this solution is diluted with water and made alkaline by the addition of sodium hydroxide solution a pale green colour, very similar in shade to that obtained with helminthosporin, is produced.

An alcoholic solution of hydroxy*iso*helminthosporin, which was orange in colour with a green fluorescence, was used after dilution with water, for the following tests.

(*a*) The addition of aqueous solutions of ammonia or sodium hydroxide gives mauve colours, which are bleached on the addition of sodium hypochlorite. Aqueous solutions of sodium bicarbonate or borax give cherry-red colours.

(*b*) Aqueous nickel sulphate solution on warming gives a rose-pink colour and later a precipitate.

(*c*) Aqueous ferric chloride solution gives a purplish-brown colour.

(*d*) Pyridine, aqueous solutions of potassium or ammonium alum and aqueous magnesium chloride solution give colour reactions similar to those given by helminthosporin.

(*e*) On shaking with solid zinc oxide, a pink colour develops, which is much more intense than that given by helminthosporin.

The dyeing properties of hydroxy*iso*helminthosporin are similar to those of helminthosporic acid, aluminium and iron mordants having been examined. The shade on aluminium is a bluer toned red-violet as compared with that produced by helminthosporic acid.

Tetra-acetylhydroxyisohelminthosporin. Hydroxy*iso*helminthosporin was boiled for a few minutes with acetic anhydride containing a few drops of sulphuric acid. The yellow solution was poured into water and the precipitated acetyl derivative was collected and crystallised from acetic acid in small, lemon-yellow rods, M.P. 234–235°. (Found: C, 60·6; H, 4·0 %. $C_{23}H_{18}O_{10}$ requires C, 60·8; H, 4·0 %.)

The acetyl derivative (1 g.) was oxidised in acetic anhydride-acetic acid solution with chromic anhydride (2 g.) in acetic acid under conditions similar to those used in the oxidation of triacetylhelminthosporin. Unchanged tetra-acetylhydroxy*iso*helminthosporin (0·7 g.) was recovered and no other product could be isolated. The oxidation was repeated under different conditions but always with the same result.

Summary.

The dried mycelium of the plant pathogen, *Helminthosporium gramineum* Rabenhorst, which is responsible for the "leaf-stripe" disease of barley, has been shown to contain about 30 % of a mixture of two hitherto undescribed pigments for which the names helminthosporin and hydroxy*iso*helminthosporin are proposed. Helminthosporin, $C_{15}H_{10}O_5$, has been shown to be 2-methyl-4:5:8-trihydroxyanthraquinone, and a number of derivatives and breakdown products are

described. Hydroxy*iso*helminthosporin, $C_{15}H_{10}O_6$, is a tetrahydroxyanthraquinone derivative, the exact nature of which has not yet been determined, though it may be 2-hydroxymethyl-1:5:8-trihydroxyanthraquinone.

The optimum cultural conditions for the production of the two pigments by the fungus have been determined.

REFERENCES.

Adkins and Folkers (1931). *J. Amer. Chem. Soc.* **53**, 1092.
Goldberg (1931). *J. Chem. Soc.* 1771.
Kinney and Langlois (1931). *J. Amer. Chem. Soc.* **53**, 2189.
Kostanecki and Niementowski (1885). *Ber. deutsch. chem. Ges.* **18**, 253.

LXXII. STUDIES ON THE LACTIC ACID, SUGAR AND INORGANIC PHOSPHORUS OF THE BLOOD OF RUMINANTS.

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(*Received February 27th, 1933.*)

Magee [1924] observed that the R.Q. of the goat after ingestion of high carbohydrate meals was unexpectedly low, the maximum being only 0·86. This observation gave rise to the idea that fermentation products with a low combustion R.Q. such as butyrates and lactates are normally produced in the rumen and that ingested carbohydrates are partly absorbed and oxidised in these forms. The same observer [1932] also found that the blood-sugar in goats did not respond to the ingestion of high carbohydrate meals in the same way as in animals with simple stomachs. In some experiments little or no increase in blood-sugar was obtained, while in others the rise was delayed for an hour or more. Although these results appeared to be in harmony with the above conception, additional experimental evidence had to be provided before it could be regarded as correct. It was obviously important, in view of the hypothesis, to determine whether an increased influx of fermentation products into the blood-stream took place after ingestion of high carbohydrate diets. As lactic acid is of greater physiological interest than butyric acid, and as a reliable method of estimating the former is available, it was decided in the first place to study the effect of carbohydrate food on the lactic acid content of the blood. Furthermore, since lactic acid is closely associated with sugar and phosphates in carbohydrate metabolism, these substances were also included in our study.

Preliminary determinations of the blood-lactic acid after the ingestion of food, gave results so variable as to suggest that the changes observed were not directly attributable to absorption but to some other factor. That this factor was of a nervous nature was suggested by the fact that repeated experiments on the same animal gave results of increasing regularity. The line of investigation was therefore extended so as to determine whether, and to what extent, excitement was capable of causing changes in the lactic acid, phosphorus and sugar of the blood. Further, as emotion is generally associated with some degree of muscular activity, the effect of varied degrees of muscular exercise on these blood constituents was also studied.

Technique.

The animals used were adult sheep and weaned lambs taken from pasture. Blood samples were taken from the jugular vein as described by Auchinachie and Fraser [1932]. The amount of oxalate used as anti-coagulant was kept as constant as possible. Lactic acid was estimated by the Friedemann, Cotonio and Shaffer method [1927], the proteins being precipitated within 1 or 2 minutes after sampling. Inorganic phosphorus was estimated by Briggs's [1922] method, and blood-sugar by the Hagedorn-Jensen method [1920].

Influence of food.

Starvation. In the first experiment, which was carried out in summer, 2 sheep were kept in a small pen and allowed nothing but water for 7 days. Blood samples were taken daily at 9 a.m. and the lactic acid and blood-sugar estimated. The second experiment was carried out in winter and the two sheep were therefore kept in cages in a warm room. Two blood samples were taken from each daily, at 8 a.m. and 4.30 p.m., and inorganic phosphorus also was estimated. The results of the second experiment are given in Figs. 1 and 2. The lactic acid

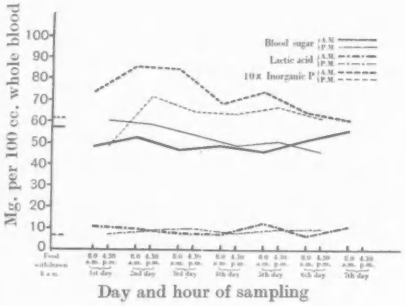

Fig. 1.

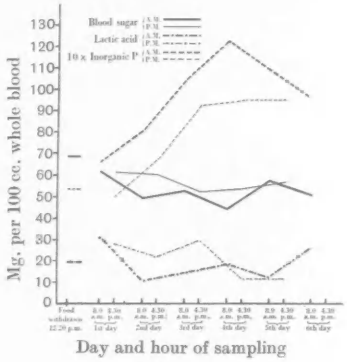

Fig. 2.

Fig. 1. Effect of starvation for 7 days. Sheep no. 8. Zero levels shown by horizontal lines on the ordinate scale.

Fig. 2. Effect of starvation for 6 days. Sheep no. 62. Zero level shown by horizontal lines on the ordinate scale.

results from the first experiment gave curves almost identical with those in Figs. 1 and 2 and they are not, therefore, shown separately. Similarly the blood-sugar in Exp. 1 varied as in Figs. 1 and 2 except that there was a fall from the 5th to the 7th day to about 15 mg. below the initial values.

The results of the second experiment show that the lactic acid curves of sheep No. 8 (Fig. 1) are relatively steady during the fast. It is noteworthy that the animal was much less excitable than No. 62 which, as can be seen (Fig. 2) gave much more irregular curves. These results show that the basal lactic acid value is reached in suitable animals after a fast of about 24 hours. This basal value of about 10 mg. per 100 cc. is approximately the same as that obtained by Carlström [1931] in horses after normal feeding, and as our own in the dog (see Fig. 4). The values from cats and kittens, obtained by Silvette and Britton [1932] are higher, averaging 25 mg. per 100 cc. after 24 hours' fast for cats and 38 mg. per 100 cc. for kittens.

The blood-sugar curves do not show as great a decrease from the 5th to the 7th day as in the first experiment, nor do they agree with the results obtained by Magee [1932], who found that in the fasting goat a general upward trend set in after 40 hours' starvation and continued until the end of the 7 days' fast. Morgulis and Edwards [1924] in studies of fasting dogs, and Lennox, O'Connor and Bellinger [1926], who studied the venous blood-sugar of normal and epileptic

subjects almost daily during starvation periods of 11 to 16 days, found that a definite hypoglycaemia developed during the 1st week, and in the 2nd week the blood-sugar tended to rise somewhat, but seldom returned to or exceeded the pre-starvation value. Our results show a tendency for the evening level to be higher than the morning level in the early days of the fast (Figs. 1 and 2).

The inorganic phosphorus curves show a definite diurnal variation, the afternoon values being always less than the morning ones. These results agree with the findings of Dupré and Semeonoff [1931]. The two phosphate curves differ markedly from each other in shape. Thus in sheep No. 8 a maximum of 8·5 mg. was reached on the 2nd day, whereas in sheep No. 62 an even and rapid rise took place from the beginning of the fast until the 4th day, when a morning maximum of 12·3 mg. was reached. The rise in the evening curve was equally sharp, but the curve ended in a plateau at a level of 9·5 mg. It may be that these differences in the phosphate curves in the two sheep are related to the fact that sheep No. 62 was much more excitable than No. 8.

Wilder [1929] has shown that in rachitic rats starvation caused serum-phosphate to increase and tetany to appear, even if the rats had previously subsisted on low phosphate diets. Similar effects due to starvation were obtained in rabbits by Gates and Grant [1927].

From the above results we concluded that the lactic acid level was constant from 24 hours onwards and, therefore, that a 24 hours' fast was sufficient to obtain a steady basal value.

Ingestion of a high carbohydrate meal.

These experiments were carried out to find the effect of the ingestion of high carbohydrate meals on the blood-sugar and lactic acid. In some of the experiments inorganic phosphorus was also determined. The following procedure was adopted. Sheep were taken in from grazing, starved for 24 hours and then fed by stomach tube with 100 g. cane sugar *plus* 250 g. maize starch in 500 cc. water. Blood samples were then taken at frequent intervals up to 10 hours after the meal. In the first few experiments a single basal determination was made before giving the food. The post-prandial curves were however very inconstant, sometimes a rise and sometimes a fall, relative to the basal value, being observed. That the irregularities were due to inconstancy in the basal level was shown by making estimations for several hours before administering the food. As a rule the first blood sample gave a higher lactic acid value than subsequent samples (see Fig. 3) and it was later found sufficient to take only three pre-experimental samples, and accept the third as the basal value. The basal phosphorus values ran parallel with those for lactic acid, whereas those for blood-sugar remained constant.

The results obtained from the various experiments were all very similar and Fig. 3 shows typical curves. Taking the third fasting sample, 7 mg., as basal, Fig. 3 shows that no significant increase in lactic acid occurred after ingestion of food. In this respect ruminants differ markedly from animals with simple stomachs, *e.g.* dogs, for it was found that the ingestion of a carbohydrate-rich meal produced in a dog a definite increase in lactic acid as well as in blood-sugar. In Fig. 4 is shown a curve typical of the results obtained in these experiments. Identical blood-sugar and lactic acid curves were obtained in the two experiments performed. These results agree with those of Ørskov [1932], who obtained a rise in blood-lactic acid after feeding pure carbohydrate to dogs, but the extent of the increase varied according to the nature of the carbohydrate given, being

higher after fructose and sucrose than after glucose. Furthermore, Campbell and Maltby [1928] detected little rise (in man) after glucose or maltose, but after 100 g. fructose or sucrose there was a very significant increase.

The effect on the blood-sugar of ingestion of carbohydrate is not so marked in the ruminant as in other animals. The amount of increase is variable and small compared with what would be expected in other mammals after ingestion of a

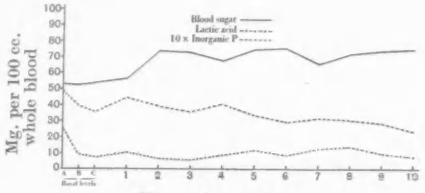

Fig. 3.

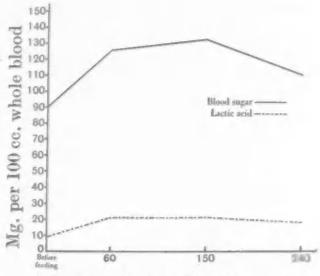

Fig. 4.

Fig. 3. Effect of carbohydrate meal (100 g. cane sugar and 250 g. starch in 500 cc. water) administered by stomach tube to sheep no. 26. Previously fasted for 24 hours.

Fig. 4. Effect of a carbohydrate meal on a dog. Previously fasted for 24 hours.

corresponding amount of carbohydrate. Richter [1928] found no diurnal variation in the blood-sugar of carefully fed cows and no alteration was observed after a meal. The results (Fig. 3) agree with the curves obtained by Magee [1932].

The inorganic phosphorus results show a slight increase for the first hour after ingestion of the meal and then a gradual downward trend contrasted with the upward trend of the blood-sugar curve. This relationship is similar to that shown by the curves obtained in man by Harrop and Benedict [1924] and by Hartman and Balliger [1925].

It is clear that the evidence provided by the foregoing experiments affords no support for the view that carbohydrate foodstuffs are taken into the blood partly in the form of organic acids. Since it has been shown [Magee, 1932] that the rumen still contains food after 7 days' fasting, one might have anticipated that progressively increasing amounts of fermentation products would have entered the blood. Furthermore, the estimations were continued long enough after ingestion of the carbohydrate meals to enable significant amounts of fermentation products to enter the blood. The fact that such increases in lactic acid were not observed in these experiments, although it does not support our hypothesis, cannot be taken as evidence against it. In the first place it has to be noted that the estimations were done on systemic blood, *i.e.* on blood after it had passed through the liver, which as Cori and Cori [1929] have shown can transform lactates into glycogen. Secondly, it is quite possible that the bulk of the fermentation products set free in the rumen consists of organic acids other than lactic, *e.g.* butyric. It may be then that estimations of these rather than of lactic acid would throw light on our problem.

It is not clear why the blood-sugar of the fasting goat should, as the experiments of Magee [1932] showed, rise gradually after 40 hours, whereas in sheep

it remains almost constant. Nor is it clear why, as in these experiments, the evening blood-sugar should consistently be above the morning level. The relatively small response of the blood-sugar to carbohydrate ingestion has been commented on by Magee [1932] and Richter [1928] and our data do not throw any additional light on the significance of the phenomenon.

We observed a marked rise in blood-inorganic P in the early stages of starvation, followed by a fall. This increase, the higher level of inorganic P in the mornings than in the evenings during starvation and the rise after ingestion of carbohydrates were too marked to be overlooked, though we are unable to attach any definite physiological significance to the phenomena. It is possible that the very gradual fall in the inorganic P curve following the carbohydrate meal may have been attributable to a normal diurnal variation.

Effects of exercise.

The work of Hopkins [1921], Meyerhof [1924] and their associates on this subject is so well known that there is no need to refer to it here. That the lactic acid found in the muscles can readily diffuse into the blood is shown by the high values which can be produced by severe muscular activity [Hill, Long and Lupton, 1924; Jervell, 1928]. Himwich [1928] has shown that venous blood from all organs, except the heart and liver, contains more lactic acid than the arterial blood passing to the organs. Havard and Reay [1926] have also shown that the blood-inorganic phosphorus first rises a little above and then falls considerably below the normal value in man after vigorous unaccustomed exercise, while in trained men this fall below normal is much less marked.

The effect of exercise on the blood-sugar level in man has been studied by numerous workers [Straub, 1922; Rakestraw, 1921; Levine, Burgess and Derick, 1924; Hale-White and Payne, 1926]. From their results Peters and Van Slyke [1931] conclude that exercise stimulates glucose formation from liver-glycogen and also the utilisation of glucose by the muscles and, as one or the other process predominates, the blood-sugar is raised or lowered. Exhaustive exercise may be followed by a reaction in which the glucose-utilising activity of the tissues drops below the level maintained during continuous rest.

Violent exercise. Two black-faced Highland sheep, Nos. 11 and 20, a ram and a wether respectively, were employed, both of which had been used for experimental work on previous occasions. They were brought in from grazing at 8 a.m. and put in a pen without food. At 9.30 a.m. a pre-exercise sample was taken and then both sheep were put into an adjoining paddock and vigorously exercised by being kept on the run, No. 11 for 15 minutes and No. 20 for 30 minutes. At the end of the exercise period, each animal was returned to the pen and the blood sampled immediately, and subsequently at 15 minute intervals for the first hour, 30 minute intervals for the second hour, and thereafter at hourly intervals for 3 hours. The animals were disturbed as little as possible. This experiment, using alternate periods of 15 and 30 minutes' violent exercise, was repeated 4 times on the same sheep at weekly or fortnightly intervals. In each case similar results were obtained. It can be seen from the graphs, Figs. 5 and 6, that similar changes occurred in the lactic acid, blood-sugar and inorganic phosphorus, the increases in lactic acid and blood-sugar being roughly proportional to the period of violent exercise. The lactic acid showed a sharp rise followed by a steady decline to about the pre-exercise level which was reached in 90 to 120 minutes after the end of the exercise. Blood-sugar showed similar but less marked changes while the inorganic phosphorus showed an

initial rise followed by an equally rapid fall to a lower plateau for a period of about 30 minutes, after which a fairly sharp rise to about normal occurred.

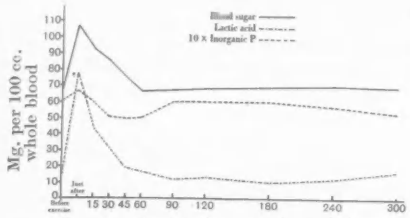

Fig. 5.

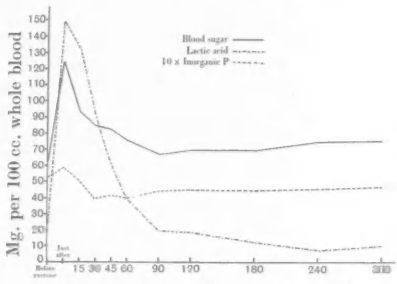

Fig. 6.

Fig. 5. Effect of 15 minutes' violent exercise. Sheep no. 11 (ram). 5. ix. 32.

Fig. 6. Effect of 30 minutes' violent exercise. Sheep no. 20 (wether). 5. ix. 32.

Moderate exercise. The effect of 15 minutes' moderate exercise was tried on 2 weaned ewe lambs which had not been previously used for experimental work. The conditions and times of sampling were the same as those just described, the only difference in procedure being that the sheep were quietly driven and not chased vigorously. A typical result is shown in Fig. 7. It can be seen that the lactic acid level is lower after the moderate exercise and remains so except for a peak at 75 minutes, which is believed to be due to the fact that several unsuccessful attempts had been made 15 minutes earlier to obtain a sample. The

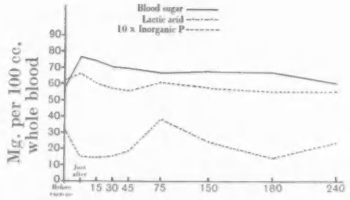

Fig. 7.

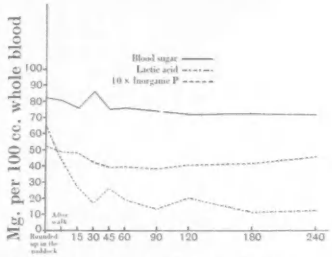

Fig. 8.

Fig. 7. Effect of 15 minutes' moderate exercise. Sheep no. 39 (ewe). 25. x. 32.

Fig. 8. Effect of 4 miles' walk under normal droving conditions. Sheep no. 20 (wether). 9. ix. 32.

blood-sugar shows a slight increase after exercise and then a gradual return to normal. The curve of the inorganic P resembles very closely those of Figs. 5 and 6.

Another experiment, the results of which are given in Fig. 8, shows the effect of moderate exercise over a prolonged period such as would be encountered in the

normal practice of droving sheep to market. Sheep Nos. 11 and 20 were again used. They were placed overnight with a flock of 40 sheep which were to be driven to market the next day. At 5.30 a.m. the flock was gathered in the paddock by the shepherd and his dog, and the two experimental animals were sampled at once. The drive of 4 miles occupied about 2 hours, the sheep travelling steadily and without undue disturbance. On arrival at the slaughter-house samples were taken and the 2 sheep placed in a separate shed and thereafter sampled at the same time intervals as before. The curves for each animal were similar, showing a higher level of lactic acid at the first sampling than at the completion of the 4 miles' drive. The lactic acid fell steadily save for a rise

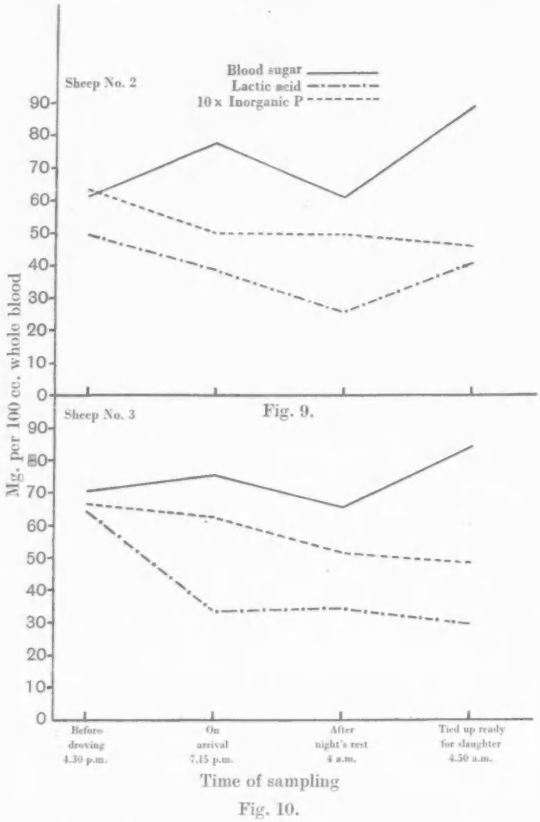

Fig. 9.

Fig. 10.

Figs. 9 and 10. Changes in blood composition of sheep driven to the slaughter-house, rested overnight and tied up for killing.

45 minutes after arrival at the slaughter-house, the blood-sugar showing a similar rise 15 minutes previously; the inorganic P showed a slight but steady depression for 90 minutes, followed by an equally steady recovery. The rise in lactic acid and blood-sugar occurred about the time when slaughtering on a lot

of cattle was commenced. The shed in which the sheep were placed was adjacent to the killing yard and the smells and noise associated with the slaughtering of animals were experienced in the pen occupied by the sheep. This rise of lactic acid and blood-sugar, coupled with the high levels obtained in the early morning after rounding-up, suggested that the excitement involved might be responsible to a large extent for these abnormal levels. Six lambs were consequently selected at random from a flock of 30 which had been sold for slaughter. They were sampled at 4.30 p.m., after being placed in a separate pen adjacent to the sheep yards. The whole flock was then driven to the slaughter-house 4 miles away, and the 6 animals again sampled on arrival about 7.15 p.m. They were left all night in a shed with no food or water and were sampled again at 4 a.m. prior to any killing operations in the slaughter-house. Slaughter then commenced, 2 of the experimental animals being killed first. After an interval of 30 minutes, during which other sheep were killed, the remaining 4 were killed. In the case of the 6 experimental sheep, final samples were taken when the animals were tied up and lying on the slaughter-table. In only 2 of the 6 sheep could the full quota of 4 samples be obtained, owing to technical difficulties, but the values obtained for Nos. 2 and 3 are borne out in essentials by the data obtained from the other sheep at each point of the experiment (see Figs. 9 and 10). The results show, as in Fig. 8, falls in lactic acid and phosphorus, but slight rises in sugar. Just before slaughtering the sugar rose distinctly and the phosphorus fell slightly in both animals, while the lactic acid rose in No. 2 but remained steady in No. 3.

It is very difficult to obtain the effect of emotion alone on an animal without that of a certain amount of exercise as well, but from the above experiments it is clear that moderate exercise, even for a longish period, without undue excitement does not raise the lactic acid level markedly.

Emotion. With a view to assessing the effect of emotion alone, 8 experiments were carried out. In 6 the procedure was as follows. 2 sheep which had been brought in from grazing and left, without food, in a pen overnight, were taken and, after being sampled for the basal level, placed in a small wire mesh cage, in which they had ample room to stand comfortably, but were not able to move more than one step backwards or forwards. A dog was made to bark at them for $1\frac{1}{2}$ or $3\frac{1}{2}$ minutes. As soon as the dog had been removed blood samples were taken every 15 minutes for the first hour, and subsequently at the time intervals shown. A typical result is shown in Fig. 11. The rise in lactic acid appears to be roughly proportional to the amount of excitement or emotion set up in the animal. Thus, sheep No. 39 was used for this class of work twice, and on the first occasion the lactic acid rose from 11 to 41·5 mg. per 100 cc., while on the second occasion, when the animal seemed much more at ease, there was no significant change apart from a slight decrease from 13·4 to 12·8 mg. per 100 cc. The highest level of lactic acid was not always found in the sample taken immediately after removal of the dog.

The general trend of the blood-sugar and inorganic P curves was similar to, though less pronounced than, that of the lactic acid curves.

In the other 2 experiments the sheep were merely prodded with a sharpened stick for 5 minutes. This stimulus apparently caused slight falls in the blood-sugar, lactic acid and inorganic P, in the case of sheep No. 39 (Fig. 12). But in sheep No. 11 a slight rise in blood-sugar and a slight fall in inorganic P occurred. The pre-emotion sample of sheep No. 39 in this experiment showed unusually high levels for all three constituents, a state of affairs which had been frequently noted in animals unaccustomed to venipuncture (see basal levels in Fig. 3). It is therefore believed that the excitement caused by the sampling was responsible

for the abnormally high basal levels (*cf.* Fig. 11), and that the emotional stimulus employed had practically no effect on these blood constituents.

From the above results it is seen that the emotional increase in lactic acid is not always accompanied by a proportional rise in blood-sugar. The curves for

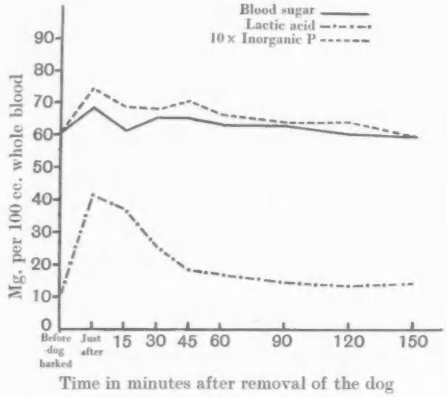

Fig. 11. Effect of dog barking for 3½ minutes. Sheep no. 39 (ewe). 4. ix. 32.

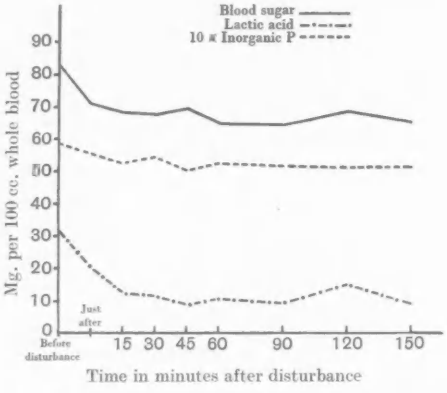

Fig. 12. Effect of prodding with pointed stick for 5 minutes. Sheep no. 39 (ewe). 1. xi. 32.

inorganic P seem to correspond more with those for lactic acid than to those for blood-sugar. These observations agree with those of Silvette and Britton [1932], who found that emotional excitement or vigorous exercise in cats produced similar effects on the blood-sugar and lactic acid.

SUMMARY.

1. The influence of fasting for 7 days, of carbohydrate meals, exercise and emotional excitement on the lactic acid, sugar and inorganic P of the blood of sheep has been studied.

2. Starvation did not appreciably alter the lactic acid or sugar content of the blood during the 7 days' fast; phosphate was always higher in the morning than in the evening, but there was a marked rise in the early stages of the fast followed by a fall.

3. The ingestion of high carbohydrate meals did not cause any significant change in the lactic acid; the effect on the blood-sugar was variable and small compared with that produced in the dog. These findings do not support the hypothesis that carbohydrates are absorbed by the ruminant partly in the form of lactates. The inorganic P increased slightly for the first hour after ingestion and then showed a gradual downward trend.

4. Vigorous exercise produced corresponding changes in lactic acid, blood-sugar and inorganic P. Increases proportional to the period of exercise occurred, the percentage increase in lactic acid being greater than for sugar. Inorganic P showed an initial rise followed by an equally rapid fall to a lower plateau, after which a fairly sharp rise to about normal occurred.

5. Moderate exercise, without undue excitement, did not increase the lactic acid. Inorganic P and blood-sugar were sometimes slightly increased, but the levels in no instance reached those obtained after vigorous exercise.

6. A significant increase in lactic acid was obtained after nervous excitement, the rise appearing to depend on the degree of excitement. The blood-sugar did not always increase correspondingly, and in nearly every case the percentage increase was greater for lactic acid. The curves for inorganic P corresponded more with those for lactic acid than with the blood-sugar curves.

We desire to express our thanks to Prof. J. J. R. Macleod and Dr H. E. Magee for advice, to Dr Magee for assistance in carrying out the experiments on the dog and on the fasting sheep, and to Mr W. Thomson for assistance with the animals.

REFERENCES.

Auchinachie and Fraser (1932). *J. Agric. Sci.* **22**, 560.
Briggs (1922). *J. Biol. Chem.* **53**, 13.
Campbell and Maltby (1928). *J. Clin. Invest.* **6**, 303.
Carlström (1931). *Skand. Arch. Physiol.* **63**, 164.
Cori and Cori (1929). *J. Biol. Chem.* **81**, 389.
Dupré and Semeonoff (1931). *J. Biol. Chem.* **94**, 341.
Friedemann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.
Gates and Grant (1927). *J. Exp. Med.* **45**, 139.
Hagedorn and Jensen (translation by Höst and Hatlehol) (1920). *J. Biol. Chem.* **42**, 347.
Hale-White and Payne (1926). *Quart. J. Med.* **19**, 393.
Harrop and Benedict (1924). *J. Biol. Chem.* **59**, 683.
Hartman and Balliger (1925). *J. Amer. Med. Assoc.* **85**, 653.
Havard and Reay (1926). *J. Physiol.* **61**, 35.
Hill, Long and Lupton (1924). *Proc. Roy. Soc. Lond.* B **96**, 438.
Himwich (1928). *Proc. Soc. Exp. Biol. Med.* **25**, 347.
Hopkins (1921). *Johns Hopkins Hosp. Bull.* **22**, 359.

Jervell (1928). *Acta Med. Scand.* Suppl. xxiv.
Lennox, O'Connor and Bellinger (1926). *Arch. Int. Med.* **38**, 553.
Levine, Burgess and Derick (1924). *J. Amer. Med. Assoc.* **82**, 1778.
Magee (1924). *J. Agric. Sci.* **14**, 600.
—— (1932). *J. Exp. Biol.* **9**, 409.
Meyerhof (1924). Chemical dynamics of life phenomena. (Philadelphia and London, Lippincott.)
Morgulis and Edwards (1924). *Amer. J. Physiol.* **68**, 477.
Ørskov (1932). *Skand. Arch. Physiol.* **63**, 213.
Peters and Van Slyke (1931). Quantitative clinical chemistry. I. Interpretations. (Baltimore and London.)
Rakestraw (1921). *J. Biol. Chem.* **47**, 565.
Richter (1928). *Biochem. Z.* **194**, 376.
Silvette and Britton (1932). *Amer. J. Physiol.* **100**, 685.
Straub (1922). *Z. klin. Med.* **93**, 123.
Wilder (1929). *J. Biol. Chem.* **81**, 65.

LXXIII. THE RELATION BETWEEN CARBOHYDRATE METABOLISM AND THE FUNCTION OF THE GREY MATTER OF THE CENTRAL NERVOUS SYSTEM.

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(*Received March 3rd, 1933.*)

It appears now to be an established fact that carbohydrate degradation plays, quantitatively, a dominant part in the metabolism of the grey matter of the brain. A number of authors have shown that lactic acid is produced from carbohydrate, and that glucose, lactic acid, and a number of other substances more or less closely related to glucose can maintain the oxygen consumption of brain tissue, *in vitro*, at a high level. (For review, see Holmes [1932, 1].) Finally, Himwich and Nahum [1932] have shown that, under most conditions, the respiratory quotient of the intact brain is unity.

An important problem, however, still awaits solution; namely, whether or not the actual functional activity of the grey matter depends directly upon carbohydrate breakdown and oxidation. It is evidently possible that, while carbohydrate metabolism is, in the long run, necessary if the neurones are to maintain their normal powers of function, activity may be immediately dependent upon some other, and hitherto unidentified, process.

The activity of peripheral nerves has been intensively studied from many points of view (see review by Gerard [1932]); we must, at least for the time being, assume that observations made on peripheral nerves apply equally to the phenomena of the central tracts; and there is nothing to suggest that either nerves or tracts are more than conductors of impulses set up by some outside machinery. It therefore seems safe to assume that the grey matter is responsible for initiating impulses, either spontaneously or in response to stimuli.

There is some evidence [Holmes, 1932, 2] which suggests that the chemical events so far identified as characteristic of grey matter may occur, not in the cells, but in the network of non-medullated processes that surrounds them and makes up the bulk of the grey matter. If this is true, then impulse initiation, if it be a function of the cell bodies, may be unconnected with carbohydrate metabolism; alternatively, of course, the cell body may serve a mere trophic function, the real seat of activity being the surrounding non-medullated network.

There is one group of phenomena which shows that normal brain function depends, ultimately, upon carbohydrate metabolism, namely the train of events known as the "hypoglycaemic response." Previous work [Holmes and Holmes, 1927; Holmes and Sherif, 1932] has shown that in hypoglycaemia the brain is suffering from actual carbohydrate starvation. But little help, however, is to be gained from a study of the subjective and objective phenomena associated with the condition. Though the final stage seems to be coma and death, all

manner of manifestations, including violent convulsions, may precede this terminal event [Wauchope, 1933]. The train of symptoms is, in any case, very far from suggesting a gradual suppression of function, such as might be expected from a gradual failure in the supply of some foodstuff directly necessary for activity. Such a suppression of function does, on the other hand, occur after the administration of anaesthetics, and here the blood-sugar is commonly raised; never, certainly, reduced. Carbohydrate oxidation by excised brain tissue can undoubtedly be reduced by anaesthetics—of the experiments showing this, the most recent are those of Quastel and Wheatley [1932, 1]. The concentration of anaesthetic employed is often high, but these authors claim (in opposition to Bülow and Holmes [1932]) to have demonstrated that such reduction occurs in the brain of the living anaesthetised animal.

It seemed likely that iodoacetic acid, which has proved of such assistance in attacking the problems of muscle physiology, might be of assistance in approaching those presented by the brain. By employing this substance, it seemed that it might be possible to discover whether the functional activity of the central nervous system could persist after the production of lactic acid from glucose had been suppressed. Krebs [1931] had previously shown that iodoacetate did, in fact, inhibit glycolysis by the cerebral grey matter, and that this inhibition resulted in a diminution of oxygen uptake. Similar results, with fluoride, had previously been obtained by Holmes [1930]; Kinnersley and Peters [1930] observed a diminution in the lactic acid content of the brains of pigeons killed with iodoacetic acid. Haldi [1932] reports that the *post mortem* lactic acid production in the brains of anaesthetised dogs, killed after (sometimes by) intravenous injections of iodoacetic acid, is sometimes, but not invariably, lowered or abolished.

In the present work, the lactic acid content of the central nervous system of frogs injected with iodoacetic acid has been found to have been reduced by some 80 %. It was observed, however, that both with frogs and with mammals death occurred fairly rapidly owing to the effect of iodoacetic acid on the heart. The circulation failed (in the mammalian experiments the blood pressure dropped suddenly to zero) and the ventricles, when examined, were found to be bloodless, pale and tightly contracted. (This recalls the work of Clark Eggleton and Eggleton [1932] on the frog's heart: these authors used isolated ventricles and found that rigor was quickly caused by oxygen lack in the presence of iodoacetic acid. The heart working *in situ* would, of course, make greater demands on the oxygen supply than would the isolated ventricle.) The existence of this rapid cardiac effect complicated the technique of the experiments here to be discussed, since it was evident that a perfusion method would have to be employed. Some preliminary work has already been done with mammals, which will be continued; for the moment, however, the problem has been approached by the easier route offered by perfusion experiments with frogs.

Von Ledebur [1932] has already carried out experiments on these lines. His results will be referred to again later; for the moment it will suffice to say that he concludes that perfusion of decerebrate frogs with Ringer solution containing iodoacetic acid abolishes the reflex excitability of the spinal cord. The technique employed was in most respects similar to von Ledebur's. (His paper was seen only after the work had been in progress for some months.) The frogs were decerebrated by cutting across the skull with scissors. They were perfused through the conus arteriosus with Ringer solution. The reservoirs of fluid were placed some 20 cm. above the cannulae, and the gas escaped to the air through a layer of mercury 0·5 cm. deep, thus ensuring a very rapid perfusion. In the

earlier experiments, the fluid contained 0·01 % $NaHCO_3$; after the oxygen had bubbled through it, the p_H was 8·0. For the majority of experiments, a Ringer solution was made according to the formula of Barkan, Broemser and Hahn [1921] and saturated with a mixture containing 97·5 % O_2 and 2·5 % CO_2. The p_H, measured by quinhydrone electrode, was 7·24. When iodoacetic acid was to be added to the perfusion fluid, a ligature was passed tightly round the hind-legs of the frog so as to include all the tissues except the sciatic nerve; this prevented the muscles of the hind-limbs from being poisoned by iodoacetic acid and left them free to contract as a result of a stimulus reaching them through the sciatics.

The method of testing whether or not the cord was active gave rise to some difficulty. Electrical stimuli are unsatisfactory in such conditions, for all the tissues are soaked in Ringer solution, and a response can always be obtained by increasing the strength of the current, since the latter will, if sufficiently strong, leak directly to the muscles. Mechanical stimuli, it was felt, were too variable in their effect. The method finally adopted was to inject 1 mg. of strychnine hydrochloride into the perfusion fluid, with a hypodermic needle, through the rubber tubing close to the cannula. If the animal then responded, by extensor spasms, to a mechanical stimulus, the cord was clearly still active, and the result was taken as a positive one. If only twitches, without sustained spasms, appeared, the result was classified as "doubtful." If no movements of the hind-limbs occurred, the response of the sciatics to electrical stimulation was tested, in case there had been a block in peripheral nerve conductivity. Only occasionally, in fact, did this seem to be the case, and it could commonly be accounted for by obvious mechanical injury to the nerves. If the sciatics were responsive to stimuli, it was assumed that the function of the cord had been abolished. The method of strychnine injection is admittedly a crude one, since a result was considered "positive" if the animal showed any extensor spasms, while, in fact, some frogs obviously convulsed for longer periods and more violently than others. It was not, however, practicable to differentiate quantitatively on these lines. In addition to observations on the activity of the cord, the amount of lactic acid in the central nervous system was estimated by pooling the central nervous systems of four frogs, excised at the end of the experiment, and working them up with trichloroacetic acid and subsequently estimating the lactic acid by the method of Friedemann, Cotonio and Shaffer [1927], the copper-lime precipitation, however, being omitted in many of the experiments for a reason to be mentioned later. The results obtained have a general bearing on the problem, which may be mentioned at once.

The lactic acid content of the frog's central nervous system, determined under these conditions, depends of course on the relative speed of three processes—lactic acid production by the tissue, the diffusion of lactic acid into the perfusion fluid and lactic acid oxidation by the tissue. The results obtained make it clear that the amphibian central nervous system, unlike the mammalian, produces lactic acid from sources other than the blood-sugar. This has, of course, previously been implied by the findings of Winterstein and Hirschberg [1925]. It probably also accounts for the very slow rate at which frogs react to insulin [Barlow, Vigor and Peck, 1931]. The perfusion fluid in these experiments contained no glucose, so that any lactic acid produced must have originated from stored precursor. At the end of 1 hour's perfusion, the lactic acid content was the same as that of the unperfused central nervous system. This shows that the lactic acid which had diffused or been oxidised must have been replaced by lactic acid newly formed in the tissue. In addition, while the central

nervous systems of frogs perfused with Ringer solution containing iodoacetic acid through which hydrogen is bubbled instead of oxygen have a very low lactic acid content, those perfused with hydrogenated Ringer solution without iodoacetic acid have a content a little higher than those treated with oxygenated Ringer solution. Evidently (since in both cases diffusion will occur, presumably at the same rate) lactic acid is replaced by fresh production in the absence of iodoacetic acid, but not in its presence. In the absence of an outside supply, the precursor must have been contained in the tissue.

These experiments do not offer any actual proof of the power of the central nervous system to oxidise lactic acid; this has, however, been so frequently demonstrated that it may be taken for granted. Table I shows the results

Table I. *Lactic acid content of central nervous system of frogs, mg./100 g. of fresh tissue.*

		After perfusion (1 hour)		
	Before perfusion	Oxygenated Ringer solution	Hydrogenated Ringer solution	Hydrogenated Ringer solution and I.A.A. 1/3000
	142	167	186	26
	145	144	137	23
	174	209	187	
	126	142	168	
	112	151	117	
	206	123		
	125	125*		
	150	98*		
	134			
Mean	146	145	159	24·5

* Perfused for 1¼ hours.

referred to above. The experiments on the effect of iodoacetic acid on the function of the central nervous system and on its lactic acid content gave the following results. The effect of iodoacetic acid on the function of the cord, as judged by the appearance or non-appearance of strychnine convulsions, was first tested by perfusion with Ringer solution containing neutralised iodoacetic acid at different concentrations. A fixed time of perfusion—75 minutes—was arbitrarily chosen. It at once became clear that there was no concentration of iodoacetic acid above which function was sharply abolished. By using a number of animals, however, and by observing the percentage which reacted, by convulsing, to strychnine after perfusion with any given concentration of iodoacetic acid, it was possible to obtain quite definite results. Eight frogs were used for each experiment, and each experiment was itself performed at least twice, so that each point on the curve represents, usually, results obtained from 16 animals. The results of the lactic acid determinations were striking. Iodoacetic acid in high dilution 0·001 %, definitely decreases the amount of lactic acid present in the central nervous system. A concentration of 0·0133 % reduces it to a low level, and further increases in concentration, even to a strength of 0·2 %, fail to depress it further, so that one suspects that the small residue of acid remaining (about 12·5 mg./100 g.) must either be produced from some different source, and one not affected by iodoacetic acid, or possibly, even, may not be lactic acid at all.

At an iodoacetic acid concentration of 0·013 %, at which the lactic acid has reached its permanent low level, 25 % of the frogs still convulse, and at

0·06 % convulsions are shown by some 12·5 %. Not, indeed, until a concentration of 0·1 % is reached, does activity seem to be finally suppressed, and, in fact, with two animals, brief convulsions have been observed with a concentration of 0·2 %. These results are shown in Fig. 1 and Table II.

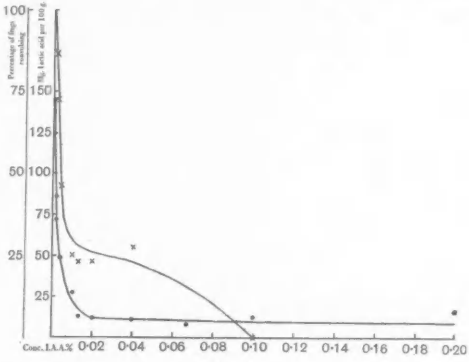

Fig. 1.

•——• Lactic acid mg./100 g. ×——× % frogs convulsing.

Table II. *Frogs perfused for 75 min. with Ringer solution containing bicarbonate 0·1 %, and saturated with 97·5 % O_2 + 2·5 % CO_2. p_H = 7·2.*

I.A.A. neutralised with sodium hydroxide.

Conc. of I.A.A.	Lactic acid mg. per 100 g.	% of frogs convulsing	No. of frogs used
0	145	100	32
0·001	86	87	16
0·002	73	73	16
0·004	48	47	16
0·01	27	25	16
0·0133	13	23	24
0·02	12	23	16
0·04	12	27	16
0·067	9	12·5	16
0·10	12	0	12
0·20	15	0*	20

* In one experiment, not forming part of this series, two frogs did show very brief convulsions after 1 hour's perfusion with 0·2 % iodoacetic acid.

Although there is a superficial agreement between the rate of suppression of lactic acid production and that of disappearance of function, as the concentration of iodoacetic acid is increased, closer scrutiny suggests that the agreement may be more apparent than real.

It is often stated [*e.g.* Quastel and Wheatley, 1932, 2] that the effect of iodoacetic acid depends upon the time during which the tissue is exposed to its action. With this in view, it was decided to see whether the effects upon function and lactic acid production could be more clearly dissociated in point of time than they could be in point of concentration of iodoacetic acid. Experiments, therefore, were performed in which the concentration of iodoacetic acid was fixed, but the frogs were perfused for 20, 40, 75 and 100 minute periods respectively. The results are shown in Figs. 2 and 3. The lactic acid is reduced, by

the end of 20 minutes, to a low level, where it remains. The reduction is somewhat greater in the presence of 0·04 % iodoacetic acid than in the presence of 0·01 % iodoacetic acid. But, in both cases, all the frogs are capable of convulsing at the end of 20 minutes; the disappearance of function is a gradual affair, and indeed, function is by no means abolished even after 75 minutes' perfusion. The rate of disappearance of function does not differ greatly with the two concentrations employed.

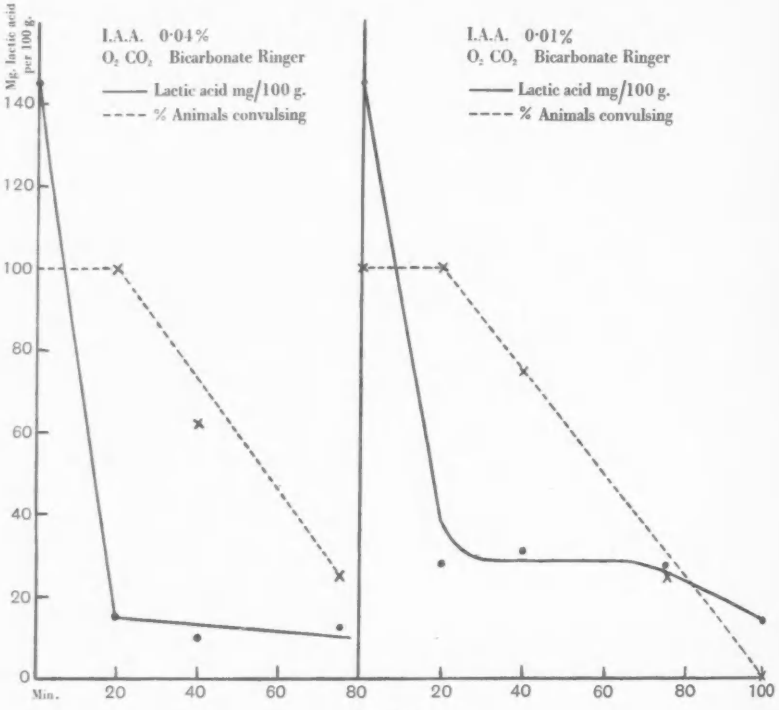

Fig. 2.

This dissociation in time seems to suggest very strongly that the two effects vary independently—in other words, however the function of the central nervous system may ultimately depend upon lactic acid formation or lactic acid oxidation, immediately it does not do so.

It must, of course, be remembered that the lactic acid estimated is that present in the central nervous system at the moment at which the perfusion is stopped, and its amount is the resultant of the three processes previously discussed—production, and removal by oxidation and diffusion respectively. The possibility, therefore, clearly cannot be ignored that, in spite of the presence of the iodoacetic acid, lactic acid is still being produced and oxidised at a rate sufficient to allow of activity being maintained. If the low figures consistently found after perfusion with iodoacetic acid do in truth represent lactic acid,

then there is ground for thinking that production and removal may still go on at a constant rate. It then, however, becomes difficult to account for the gradual and more slowly occurring failure in function. If there is enough lactic acid production and oxidation to maintain function at the end of 20 minutes, why should it not persist unimpaired for 75 minutes, since during that time there is no evident change in the level of lactic acid? A more probable explanation seems to be that function is directly connected neither with the production nor with the oxidation of lactic acid, but depends on some process which is more slowly suppressed by iodoacetic acid. The situation, unfortunately, is not clarified when one examines the effect of adding lactate to the perfusion fluid. As has just been pointed out, it is still conceivable that function may depend on the oxidative removal of lactic acid. If, however, sodium lactate is added to the perfusion fluid together with iodoacetic acid, the former in increasing concentrations, the effect of the iodoacetic acid in abolishing function is not in any way modified until concentrations as high as 0·5 % of lactate are reached. There is then a partial reversal of the iodoacetic acid effect, which, however, is evident even with high (0·2 %) concentrations of iodoacetic acid. If this result is due merely to the presence of lactate in the central nervous system, there is no obvious reason why it should not occur with much lower concentrations of lactate in the perfusion fluid, which, it is easy to show, raise the lactic acid content of the central nervous system to a marked degree. These points are illustrated in Table III.

Table III. *Perfused with O_2/CO_2 bicarbonate Ringer.* p_H *7·24.*

I.A.A. neutralised with sodium hydroxide.

Conc. I.A.A. %	Conc. lactate %	Lactic acid in C.N.S. mg./100 g.	Frogs convulsing / total used	Time min.
0·2	0	14	$\frac{1}{4}$	60
0·2	0·25	110	$\frac{4}{7}$	60
0·2	0·5	175	$\frac{2}{4}$	60
0·2	0	17	$\frac{0}{24}$	75
0·2	0·5	171	$\frac{4}{14}$	75

Feng [1932] has recently claimed that the abolition of conduction in peripheral nerve, which occurs as a result of treatment with iodoacetic acid, can be delayed by the immersion of the nerve in solutions containing lactate. He found it necessary to use 0·4 % iodoacetic acid, that is double the maximum concentration here employed. Possibly, therefore, the two phenomena may be unrelated. Moreover, in the present work, the excitability of the sciatics was tested at the end of each experiment, and the presence of a nerve block thus excluded. Gerard has, however, stressed the relative impermeability of the nerve sheath to many substances; and this impermeability may account for the high concentrations of iodoacetic acid which Feng found to be necessary. No such connective tissue sheath surrounds the white medullated fibres of the cord, which may therefore be more accessible to that substance.

In the present experiments, both cord and the upper portions of the sciatic nerves were perfused, and care was taken to dissect away the urostile and to stimulate the nerves close to their exit from the sacrum, since the procedure of tying off the legs prevents the perfusion fluid from reaching the lower portions of the nerves. Gerard (personal communication) has confirmed Feng's findings, and has shown, in addition, that added lactate will maintain the oxygen uptake

of nerve poisoned with iodoacetic acid, though it does not affect that of normal nerves. The fact that the failure, both of conductivity and of oxygen uptake, is gradual is in accordance with the low metabolic rate displayed by nerves. Von Ledebur [1932] also found that added lactate maintained the oxygen uptake of the frog's spinal cord after treatment with iodoacetic acid.

In view of the recent work of Winterstein [1930] the possibility cannot be entirely ignored that nerves may be sensitive to electrical stimulation when they are insensitive to stimuli originating in the grey matter. Apart from this, however, and considering the rest of the evidence together (bearing in mind, too, the fact that the spinal cord has a much richer vascular supply than peripheral nerve, so that probably the tracts in the cord are more sensitive to perfusion with iodoacetic acid than are the sciatic nerves), it seems possible that the failure in function observed really represents a failure in conduction, partly or wholly central (*i.e.* in the tracts of the cord). But if this is so, the probability seems to be strengthened that the grey matter itself is less sensitive even than appears from these experiments to the cessation of lactic acid production, and to its absence from the rôle of a substrate for oxidation, for otherwise, with its relatively high metabolic rate[1], one would expect an early and complete cessation of its function under the influence of iodoacetic acid.

The effect of substances other than lactic acid upon the function of the central nervous system has been tested. Pyruvate, succinate and (as was to be expected) glucose exert no protective effect against I.A.A. Methylglyoxal, however, has some potency in this respect and at lower concentration than lactate. In higher concentration (*e.g.* 0·5 %) it is, in fact, itself toxic, since it throws the whole animal into rigor. The methylglyoxal used was prepared from *iso*nitrosoacetone, made from ethyl acetoacetate according to the directions of Charrier [1907]. The methylglyoxal was prepared by distilling the *iso*nitrosoacetone with nitrosylsulphuric acid [Hoffman and Neuberg, 1930], a 1–2 % solution being obtained. The strength was determined by treatment with a known amount of hypoiodite and back-titration with thiosulphate. In addition, a few determinations were made by means of the *p*-nitrophenylosazone.

Estimations of lactic acid were made on the central nervous system of frogs perfused with methylglyoxal and iodoacetic acid. If the copper-lime technique is employed, a large amount of lactic acid is produced from unchanged methylglyoxal. The copper-lime procedure was therefore omitted, without any alteration in the values obtained when methylglyoxal was not used. A similar device has been resorted to by Haarman [1932].

These estimations show that, in the presence of methylglyoxal, there is a small increase in the amount of lactic acid in the central nervous system. The increase is much less than that obtained by perfusion even with concentrations of lactate that have no effect in preventing the inhibition of convulsions by iodoacetic acid. Its effect is clearly, therefore, not due merely to an increased production of lactic acid, which, perhaps, is the first solution that suggests itself. Indeed, it introduces a further complication. Dudley [1931] has presented strong evidence that the action of iodoacetic acid in muscle is to prevent the production of lactic acid by inhibiting the enzyme glyoxalase, which converts

[1] It is worth noting, for instance, that Hoffmann, Holzlöhner and Leegaard [1932] find that the frog's spinal cord produces heat at the rate of 580–2324 microcalories per g. for each second of stimulation. For medullated nerve, at 20°, the rate appears, from Hill's [1932] data, to be some 160 microcalories per g. for 1 second's stimulation. Since a large portion of the spinal cord must be white matter, the heat production of grey matter must be very large indeed compared with that of white.

methylglyoxal into lactic acid. Even with high concentrations of iodoacetic acid, some slight increase occurs in the lactic acid of the central nervous system, but it seems more reasonable, in view of Dudley's results, to suppose that methylglyoxal is effective by virtue of the fact that it follows some other metabolic pathway.

Table IV.

Conc. I.A.A. %	Conc. methylglyoxal %	Lactic acid in C.N.S. mg./100 g.	Frogs convulsing / total used	Time of perfusion (min.)
Frogs perfused with O_2/CO_2 bicarbonate Ringer p_H 7·2:				
0·2	0·05	26	$\frac{3}{3}$	60
0·1	0·05	22	$\frac{3}{4}$*	75
0·1	0·06	21	$\frac{3}{4}$	75
0·1	0·06	22	$\frac{0}{4}$	75
Frogs perfused with oxygenated bicarbonate Ringer (no CO_2) p_H 8·0:				
0·0333	0·034	66	$\frac{3}{4}$	60
0·0333	0·06	57	$\frac{4}{4}$	60
0·0333	0	24	$\frac{1}{4}$	60
0·05	0·05	34	$\frac{2}{4}$	60
0·05	0	12	$\frac{0}{4}$	60
0·05	0·05	29	$\frac{2}{4}$	60
0·05	0	8	$\frac{1}{4}$	60

* All showed marked twitchings. No actual convulsions.

Lohmann [1932] has found that glutathione is a coferment for the glyoxalase reaction, and Quastel [1933] and Dickens [1933] that iodoacetic acid inhibits this action of glutathione. An attempt, however, to restore the function of the central nervous system previously destroyed by 1 hour's perfusion with 0·2% iodoacetic acid, by subsequent perfusion with glutathione (0·05 %) and with glutathione 0·05 % *plus* methylglyoxal 0·05 % was unsuccessful: but the point clearly needs further investigation.

The dissociation in time between the depression of lactic acid production by the central nervous system and failure in its function makes it possible that, as in muscle, some other mechanism, such for instance, as the breakdown of phosphocreatine, may intervene between the two processes. The nerve cells may be able to function for a time without lactic acid, just as the muscle can contract without it, because, as in muscle, function may depend directly upon the breakdown of phosphocreatine and only indirectly on the formation or oxidation of lactic acid. An obvious step, therefore, was to investigate the behaviour of labile phosphate in the presence of iodoacetic acid. Gerard and Wallen [1929] have found that peripheral nerve contains labile phosphate and Gerard and Tupikow [1930] that it contains labile creatine; these are in equivalent amounts, and there is no reasonable doubt that they represent phosphocreatine. Stimulation of the nerve causes phosphocreatine to break down, and Gerard [1932] has recently discussed the evidence suggesting that the process is connected with impulse propagation. The next step seemed, therefore, to be to investigate the creatine phosphate content of the frog's central nervous system, and to try to discover what the effect upon it of iodoacetic acid might be. To this end, labile phosphate was estimated by the method of Eggleton and Eggleton [1929], the only important modification being that the PO_4 was estimated by the method of Fiske and Subarrow [1925] instead of by that of Briggs. The figures for the content of the central nervous system, removed immediately after decerebration, are given in Table V. As the central nervous systems were dissected out, they were quickly weighed and placed in a beaker containing

Table V. *Labile phosphate, expressed as mg. P/100 g. in C.N.S. of frogs removed immediately after decerebration.*

No. of frogs	Species	Labile P, mg./100 g.
8	English *temporaria*	3·92
12	,,	3·24
6	Hungarian *esculenta*	5·01
4	,,	3·80
4	,,	3·23
	Mean	3·84

iced, oxygenated Ringer solution, to minimise the chance of creatine phosphate breaking down during the period of 7–10 minutes necessarily consumed by dissecting eight or more central nervous systems. To minimise this delay, large Hungarian frogs, four of which yield enough tissue for estimation, were used in some experiments. The amount of labile PO_4 was extremely low, and the colours available for comparison in many instances were so pale that the results cannot be regarded as more than approximate.

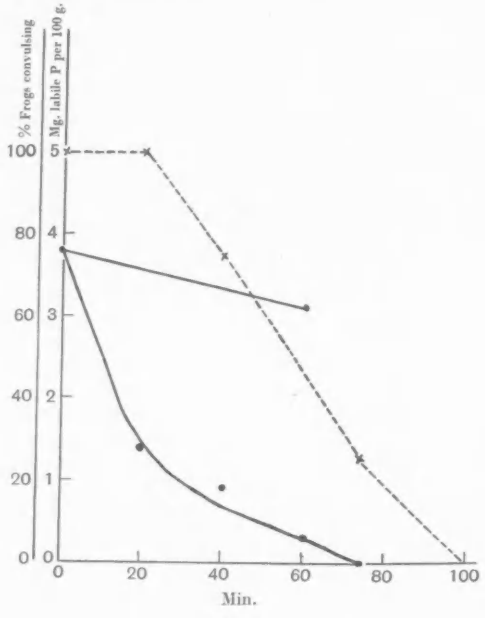

Fig. 3.

I.A.A. 0·01%. O_2/CO_2 bicarbonate Ringer. —— Labile P mg /100 g. ---- % animals convulsing. Upper continuous line represents effect of perfusion without I.A.A.

Perfusion of the central nervous system for 1 hour, without the addition of iodoacetic acid and without the administration of strychnine, caused a decrease in the content of labile P, as is shown in Table VI. All these animals, of course, were very active at the end of the perfusion.

The effect of iodoacetic acid on the labile P is to cause a diminution in its amount. The figures obtained are shown in Table VII and Fig. 3. The "activity" curve is the same in Fig. 2 (0·01 % iodoacetic acid), and there is considerable

Table VI. *Frogs perfused for 1 hour with O_2/CO_2 bicarbonate Ringer solution.*

No iodoacetic acid. No strychnine.

No. of frogs	Species	Labile P, mg./100 g.
10	English *temporaria*	2·92
10	,,	4·26
10	,,	2·50
10	,,	3·05
	Mean	3·18

similarity between the fall in labile P, shown in Fig. 3, and that of lactic acid as shown in Fig. 2. The amount of labile P has become undetectable before function has disappeared, and there seems no more evidence in favour of activity depending directly on the presence of creatine phosphate than there is that it is associated directly with lactic acid production or oxidation.

Gerard has lately found (personal communication) that the creatine phosphate content of nerves soaked in Ringer solution containing lactate falls more slowly than it does if the nerves are kept in lactate-free Ringer. It will be seen from Table VII that, after 40 minutes' perfusion with 0·01 % I.A.A. and 0·5 % lactate, the creatine phosphate content of the central nervous system is higher than after perfusion by I.A.A. alone.

Table VII.

Time of perfusion min.	I.A.A. 0·01 %. No strychnine injected	Perfused with I.A.A. 0·01 %. Strychnine injected	I.A.A. 0·01 % + Na lactate 0·5 %. Strychnine injected
20	1·30 *E*	1·59 *E*	—
40	0·949 *T*	0·596 *T*	1·10 *T*
	0·728 *T*	—	1·24 *E*
	0·959 *T*	—	—
60	0·10 *E*	0 *E*	1·38 *E*
		0·40 *T*	0·89 *T*
75	0	—	—

T indicates English *temporaria*. *E* indicates Hungarian *esculenta*.

In view of Gerard and Wallen's figures for the creatine phosphate content of nerve, it seems an open question whether the creatine phosphate content of the central nervous system may not, in fact, be confined to the white matter. It is unlikely that the medullated tracts differ from nerve in respect of their content of the substance, yet the average figure for the frog's central nervous system is 3·84 mg./100 g. while, according to Gerard and Tupikow [1930], that for frog's nerve is about 9·5 mg./100 g. It is difficult, if not impossible, accurately to decide the relative proportions of grey and white matter in the frog's central nervous system, and the figure given here for its labile phosphorus content is certainly an approximate one; still, it is evident that if the amounts of grey and white matter were equal the figures would at least be consistent with the

possibility that the creatine phosphate content of grey matter is zero, while that of white matter is similar to that of nerve. Mair and Lorrain Smith [1912] found that the dried white matter of adult human brain contained 60 % of chloroform-soluble substance, while the dried grey matter contained 30 %. If one is prepared to make the (doubtful) assumption that these figures hold good for the central nervous system of the frog, it would be possible to calculate the relative proportions of grey and white matter present from the percentage of chloroform-soluble substance present in the whole tissue, after it had been dried. Two determinations, for each of which eight central nervous systems were used, gave the following figures. Dry weight as percentage of wet weight, 19·8, 18·4. Chloroform-soluble substance as percentage of dry weight, 46·8, 45·6; mean 46·2. This gives a ratio of grey matter/white matter of 0·852:1. If all the labile phosphorus is in the white matter, then its concentration is 9·01 mg. per 100 g., a figure close to that obtained by Gerard for nerve. I have no wish to emphasise this figure, however, for it is founded on a very questionable assumption and on determinations which, owing to the small quantity of material available, cannot be accurate.

Just before this paper was written, the author had the privilege of reading, in typescript, a paper by Chang and Gerard, the contents of which have already twice been referred to as a "personal communication." The authors describe experiments showing that the addition of lactate to peripheral nerve will much delay, though it will not permanently prevent, the diminution in oxygen consumption and power of conduction and the increase in phosphocreatine breakdown, which occur as the result of treatment with iodoacetic acid[1]. These results are, in many respects, very suggestive of those which are described in this paper. The partial reversal of the effect of iodoacetic acid on the capacity of the central nervous system to respond to strychnine by the addition of lactate to the perfusion fluid, might well be ascribed to an incomplete protection of the medullated tracts against the effect of the poison. It was found, it is true, that the sciatics responded to electrical stimulation at the end of the experiment, but the experiments were all of much shorter duration than were Gerard's (60–75 minutes for the most part, as against 2½ to 12 hours). As has been earlier suggested, it is quite conceivable that the larger vascular supply of the cord renders its tracts far more accessible to perfused iodoacetic acid than are peripheral nerves, even when they also are perfused. If, however, the failure which is observed is a failure of conduction in the tracts, it may well be that the grey matter itself may, in reality, have continued to be capable of function for a longer time even than these experiments suggest.

There seems to be no doubt that lactic acid production, in the frog's central nervous system, is carried on at the expense of precursor stored in the tissue itself, and not, as in the mammalian central nervous system, at the expense of the blood-sugar. This may or may not indicate a fundamental difference in the chemical machinery underlying function in the two types, but at least it makes it certain that the state of affairs cannot be assumed to be the same in the two instances without further investigation.

[1] The experiments of Chang and Gerard, as well as those of Feng, raise the question as to whether nerve cannot, after all, oxidise lactic acid. In this connection, it is worth noting that Holmes [1930] found that the white matter of the central nervous system could do so to a slight extent. At the time, this result, in view of those previously obtained with nerve, seemed inexplicable; but in the light of these recent findings it becomes of more interest.

SUMMARY.

1. The capacity of the frog's central nervous system to respond to strychnine injections by convulsions is abolished by iodoacetic acid, but perfusion for $1\frac{1}{4}$ hours with 0·1 % iodoacetic acid is necessary for complete abolition. Perfusion for the same period with a concentration of 0·01 % reduces the lactic acid content to a very low level.

2. The capacity of the central nervous system to respond to strychnine is intact after 20 minutes' perfusion with 0·04 % iodoacetic acid, at which time the lactic acid content has been reduced almost to the minimum level reached.

3. The abolition of central nervous system function by iodoacetic acid is partially prevented by the presence of 0·5 % sodium lactate and 0·05 % methylglyoxal.

4. The "labile phosphorus," presumably representing the phosphocreatine, of the central nervous system, is reduced by iodoacetic acid more rapidly than the function of the tissue is affected.

5. It is suggested that the effect of iodoacetic acid may be chiefly on conduction in the white matter, and that the activity of the grey matter depends immediately neither on phosphocreatine breakdown, nor on lactic acid formation or oxidation.

I wish to thank Mr T. C. Fletcher for his careful assistance with these experiments. I am indebted to the Government Grant Committee of the Royal Society for a grant covering part of the expenditure on animals.

REFERENCES.

Barkan, Broemser and Hahn (1921). *Z. Biol.* **74**, 1.
Barlow, Vigor and Peck (1931). *J. Pharm. Exp. Ther.* **41**, 229.
Bülow and Holmes (1932). *Biochem. Z.* **245**, 459.
Charrier (1907). *Chem. Zentr.* II, 231.
Clark, Eggleton and Eggleton (1932). *J. Physiol.* **75**, 332.
Dickens (1933). *Nature*, 130.
Dudley (1931). *Biochem. J.* **25**, 439.
Eggleton and Eggleton (1929). *J. Physiol.* **68**, 193.
Feng (1932). *J. Physiol.* **76**, 477.
Fiske and Subarrow (1925). *J. Biol. Chem.* **66**, 375.
Friedemann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.
Gerard (1932). *Physiol. Rev.* **12**, 469.
—— and Tupikow (1930). *Proc. Soc. Exp. Biol. Med.* **27**, 360.
—— and Wallen (1929). *Amer. J. Physiol.* **89**, 108.
Haarmann (1932). *Biochem. Z.* **255**, 125.
Haldi (1932). *Amer. J. Physiol.* **101**, 469.
Hill (1932). *Proc. Roy. Soc. Lond.* B **111**, 106.
Himwich and Nahum (1932). *Amer. J. Physiol.* **101**, 446.
Hoffmann, Holzlöhner and Leegaard (1932). *Z. Biol.* **93**, 108.
—— and Neuberg (1930). *Biochem. Z.* **226**, 490.
Holmes (1930). *Biochem. J.* **24**, 914.
—— (1932, 1). *Ann. Rev. Biochem.* **1**, 487.
—— (1932, 2). *Biochem. J.* **26**, 2005.

Holmes and Holmes (1927). *Biochem. J.* **21**, 412.
—— and Sherif (1932). *Biochem. J.* **26**, 381.
Kinnersley and Peters (1930). *Biochem. J.* **24**, 710.
Krebs (1931). *Biochem. Z.* **234**, 278.
von Ledebur (1932). *Pflüger's Arch.* **230**, 229.
Lohmann (1932). *Biochem. Z.* **254**, 332.
Mair and Lorrain Smith (1912). *J. Path. Bact.* **17**, 609.
Quastel (1933). *Nature*, 206.
—— and Wheatley (1932, 1). *Proc. Roy. Soc. Lond.* B **112**, 60.
—— —— (1932, 2). *Biochem. J.* **26**, 725.
Wauchope (1933). *Quart. J. Med.* **11**, 117.
Winterstein (1930). *Science*, **71**, 641.
—— and Hirschberg (1925). *Biochem. Z.* **159**, 351.

LXXIV. SOME PROPERTIES OF THE ENZYME GLYOXALASE.

II. p_H-ACTIVITY CURVE WITH PHENYLGLYOXAL AND THE EFFECT OF GLUTATHIONE.

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(*Received March 3rd, 1933.*)

The p_H-activity curve of glyoxalase with phenylglyoxal as substrate and phosphate buffer.

In a previously published paper [Giršavičius, 1932] an experiment on the p_H-activity of glyoxalase, acting on phenylglyoxal, was described. Diethyl barbiturate buffer was used although it inhibits glyoxalase, since earlier work by Kuhn and Heckscher [1926] had apparently shown a p_H-optimum in the neighbourhood of p_H 8.

Since however the writer's experiments indicated an optimum at p_H 6–7 the determination has been repeated with phosphate as buffer. Although phosphate also inhibits glyoxalase to a not inconsiderable degree, a more suitable buffer has not yet been discovered. Kuhn and Heckscher had used phosphate, and it seemed desirable to find out whether the discrepancy in the results described by them and by me was not at least partly due to a specific effect of one of the buffer salts.

The following experiment is in principle similar to the one described previously[1].

The solutions consisted of 200 cc. liver extract + 75 cc. 0·2 *M* phosphate buffer of varying p_H + 20 cc. phenylglyoxal solution. The phosphate buffers were prepared by mixing 0·2 *M* Na_2HPO_4 and 0·2 *M* KH_2PO_4. The phenylglyoxal was added as the last ingredient to the solutions already warmed to 37°.

The solutions were incubated for 3½ hours. Initially and at the end of the incubation period p_H estimations were done on all solutions (quinhydrone electrode).

Fig. 1 shows the results. In the shorter time the reactions have proceeded less far than in the previously published experiment, notwithstanding that the temperature is higher by 10°. The p_H shift was correspondingly less, so that the position of the p_H-activity curve, and its peak, can be more closely defined. The optimum lies between p_H 6·2 and 6·6.

[1] The polarimetric method [Giršavičius, 1932] is not very satisfactory when phosphate is present in the solutions. Some phosphate passes through the amyl alcohol and causes turbidity development in the uranyl nitrate solution. To avoid this, the alkaline extracts (containing mandelic acid) are treated with some concentrated baryta, filtered, then neutralised with dilute H_2SO_4, as previously described.

As the meaning of such double curves is not, perhaps, obvious, the following argument may be useful. The p_H of every incubated solution fell continuously, in the course of the reaction, from an initial to a final value; the total transformation in each solution can be regarded as the sum of the transformations achieved, in immeasurably short periods of time, at each intermediate p_H. If it were possible so to stabilise the acidity of the solution, that the p_H should not change in the course of the reaction, then one could also find one single p_H value at which the total transformation would be the same as actually observed. This p_H value we might call the equivalent p_H of the solution. A curve drawn through the equivalent p_H of the incubated solutions should be the actual p_H-activity curve of the enzyme (under the given experimental conditions). Where these p_H values actually lie cannot be determined from the data of the present experiment: a simple consideration shows however that the equivalent p_H of each solution must fall inside the experimentally observed p_H-span. The equivalent p_H of that solution in which the transformation has proceeded

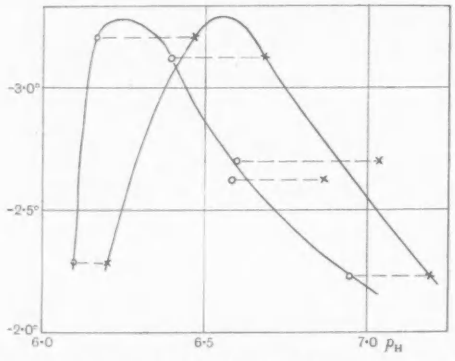

Fig. 1. p_H-activity curve of glyoxalase, with phenylglyoxal as substrate and phosphate buffer. (×) Initial, (o) final p_H-values.

furthest in the given time (here the solution in which at the end a rotation of $-3{\cdot}12°$ was found) must lie nearer to the p_H-optimum of the enzyme than the equivalent p_H of any other solution. At the same time, however, this equivalent p_H need not coincide with the p_H-optimum. The latter need not even fall within the p_H-span of the "top" solution. It would, therefore, be unjustified to assume that the p_H-optimum of the enzyme must lie between the peaks of the two curves, representing initial and final p_H. On the other hand the p_H-optimum cannot lie beyond the outside limits of the next lower p_H-span on each side. (It cannot even lie as far out as these limits, since it must lie nearer to the equivalent p_H of the "top" solution than to the equivalent p_H of any other solution.)

p_H 6·1 and 6·65 can be taken as the extreme values between which the p_H-optimum of glyoxalase, with phenylglyoxal as substrate and phosphate buffer, may lie. An inspection of the curves suggests a p_H-optimum near p_H 6·4–6·5.

The probable reason for the discrepancy between Kuhn and Heckscher's and my experiments has been indicated previously. The p_H-shift in the course of prolonged incubation of glyoxalase with a substrate can be so considerable that its neglect must lead to the apparent finding of too alkaline an optimum p_H-range.

The co-glyoxalase action of glutathione.

Lohmann [1932] has discovered that addition of reduced glutathione (GSH) powerfully activates glyoxalase, and restores its action after inhibition by dialysis. In other words, GSH functions as a co-enzyme; most probably it is the natural co-enzyme of glyoxalase. In a recent paper [Giršavičius, 1933], I am able to confirm, qualitatively, some of Lohmann's findings. A more exact quantitative investigation of the GSH effect is given in the following experiment.

The method used is essentially that described in a previous paper [Giršavičius, 1931]: phenylglyoxal was estimated by a micro-modification of Friedemann's method. Some improvements which have been introduced are described elsewhere [Giršavičius, 1933]. In the same recent paper conditions are discussed under which substrate estimation can form the methodical basis of glyoxalase investigation.

Liver extract was prepared from fresh rabbit's liver (1/6000 8-hydroxyquinoline sulphate[1]). The GSH solution, contained 5·1 mg. per cc.

Solutions (*a*)–(*g*) contained 40 cc. liver extract, 20 cc. phosphate buffer, and 15 cc. of water and GSH solution in varying proportions. Solutions (*h*)–(*j*) were similarly made up, but contained 40 cc. of water instead of liver extract, and were intended as controls, for the phenylglyoxal destruction by GSH alone.

	(*a*), (*b*)	(*c*)	(*d*)	(*e*), (*h*)	(*f*), (*i*)	(*g*), (*j*)
cc. GSH solution	0	1	2	5	10	15
% GSH	0	0·006	0·012	0·030	0·060	0·090

After warming these solutions to 38°, 10 cc. phenylglyoxal solution were added. Samples were taken immediately, and after 45 mins., 1 hour 30 mins., and 2 hours 30 mins.

The experimental results are given in Table I. In an investigation of reactions, whose course does not allow of a simple mathematical expression, the initial velocities form the most useful term of comparison. This is especially true of reactions catalysed by glyoxalase, owing to the rapid destruction of the enzyme on incubation (see next experiment). The method used by me (at the suggestion of Prof. J. B. S. Haldane) has been fully explained elsewhere [Giršavičius, 1933]. It may be briefly indicated here.

We will call the transformation accomplished in a given time (t), Δx. The experimental results present themselves as a series of values Δx_1, Δx_2, *etc.*, corresponding to the times t_1, t_2, *etc.* (*e.g.* the transformations achieved in the 1st hour, the first 2 hours, *etc.*). By plotting against the times t_1, t_2, *etc.* the corresponding mean velocities, $\Delta x_1/t_1$, $\Delta x_2/t_2$, *etc.*, a curve is obtained, whose extrapolation to $t = 0$ gives the initial velocity of the reaction (dx_0/dt_0).

In Table I the Δx values, and the $\Delta x/t$ values are given, besides the actual phenylglyoxal content of the solutions, as determined analytically. From these data extrapolation curves were constructed; the initial velocities, so obtained, are plotted in Fig. 2 against the concentration of added GSH. (Note that some glutathione is present in the liver extract to start with. The actual glutathione concentrations are therefore somewhat higher than shown.)

GSH itself reacts gradually with phenylglyoxal on incubation[2]. To obtain the actual enzyme activation, it is necessary to subtract from the curve of Fig. 2

[1] The reasons for adopting 8-hydroxyquinoline sulphate as an antiseptic, and the method of using it, have been described previously [1933].

[2] The GSH solutions developed a yellow colour on incubation with phenylglyoxal—increasing in intensity with increasing GSH concentration.

Table I.

Phenylglyoxal.

	Initial mg./cc.	After 45 min. mg./cc.	After 45 min. Δx	After 1 hr. 30 mins. mg./cc.	After 1 hr. 30 mins. Δx ($\Delta x/2$)	After 2 hrs. 30 mins. mg./cc.	After 2 hrs. 30 mins. Δx ($\Delta x \times 3/10$)
a	1·79, 1·82 → 1·80	1·61, 1·61 → 1·61	−0·19	1·48, 1·50 → 1·49	−0·31 (−0·16)	—	—
b	1·80, 1·84 → 1·82	1·64, 1·63 → 1·64	−0·18	1·49, 1·51 → 1·50	−0·32 (−0·16)	—	—
c	1·80, 1·83 → 1·82	1·39, 1·40 → 1·40	−0·42	1·25, 1·27 → 1·26	−0·55 (−0·28)	1·04, 1·04 → 1·04	−0·78 (−0·23)
d	1·76, 1·74 → 1·75	1·35, 1·35 → 1·35	−0·40	—	—	0·81, 0·83 → 0·82	−0·93 (−0·28)
e	1·65, 1·65 → 1·65	0·96, 0·97 → 0·96	−0·69	0·51, 0·54 → 0·52	−1·13 (−0·56)	0·26, 0·27 → 0·26	−1·39 (−0·42)
f	1·55, 1·54 → 1·54	0·75, 0·77 → 0·76	−0·78	0·22, 0·25 → 0·24	−1·30 (−0·65)	0·04, 0·06 → 0·05	−1·49 (−0·45)
g	1·37, 1·42 → 1·40	0·58, 0·56 → 0·57	−0·83	0·12, 0·12 → 0·12	−1·28 (−0·64)	—	—
				Control solutions.			
h	1·81, 1·84 → 1·82	—	—	1·64, 1·67 → 1·66	−0·16 (0·08)	1·55, 1·55 → 1·55	−0·27 (−0·08)
i	1·87, 1·88 → 1·88	—	—	—	—	1·68, 1·68 → 1·68	−0·20 (−0·06)
j	1·88, 1·88 → 1·88	—	—	1·72, 1·71 → 1·72	−0·16 (0·08)	1·72, 1·72 → 1·72	−0·16 (−0·05)

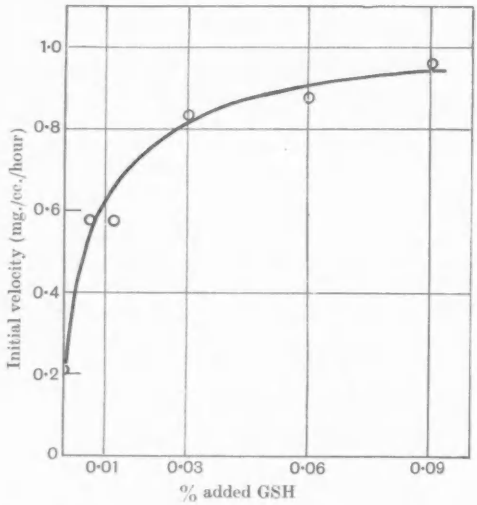

Fig. 2. Velocity of phenylglyoxal destruction by liver extract as a function of the concentration of added GSH.

the phenylglyoxal destruction by GSH alone. As the Table shows, the results of the control experiments (*h*)–(*j*) are unsatisfactory; at any rate the effect is small enough to be neglected without serious error.

Lohmann observed maximum activation both of rat's liver- and rat's muscle-glyoxalase at GSH concentrations between 0·02 and 0·03 %. In the present experiment a concentration 3–4 times as high (0·08–0·1 %) seems necessary for full activation. Whether the different substrate accounts for the different result remains to be seen.

An inspection of the figures of Table I shows that the initially determined phenylglyoxal concentration decreases as the GSH concentration in the solution increases. This phenomenon, the initial glyoxal disappearance caused by glutathione, has been discussed in another paper [Giršavičius, 1933]. It is observed whenever the GSH concentration in a solution is increased before adding phenylglyoxal.

The identification of reduced glutathione as the co-enzyme of glyoxalase raises the question as to the effect of presence or absence of oxygen on the stability of the enzyme. Lohmann has shown that aeration, sufficient to oxidise the GSH, inactivates glyoxalase. It seemed desirable to investigate the possible effect of oxygen under experimental conditions similar to those obtaining in my other experiments, and with special reference to antiglyoxalase action.

In the following experiment liver extract was incubated with and without pancreatin, under conditions of anaerobiosis and of moderate aerobiosis (incubation in a thin layer). At intervals the glyoxalase activity of the solutions was tested.

Liver extract, 2 days in refrigerator, containing 1/4200 8-hydroxyquinoline sulphate: pancreatin solution, 40 mg. pancreatin (Park, Davis and Co.) per 100 cc.

(*a*) Liver extract, 5 cc. water to 100 cc.

(*b*) Liver extract, 5 cc. pancreatin solution to 100 cc.

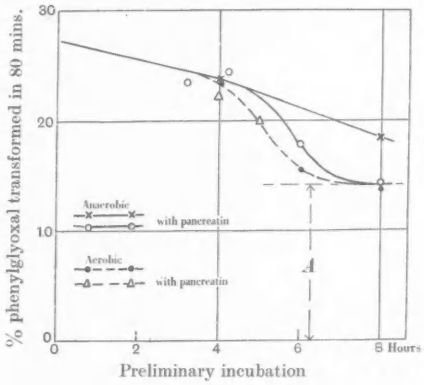

Fig. 3. Fall in glyoxalase activity on incubation, aerobically and anaerobically, with and without pancreatin. *A*, phenylglyoxal destruction after glyoxalase inhibition (non-enzymic reactions).

For anaerobic incubation 25 cc. (*a*) or (*b*) were measured into 75 cc. Thunberg tubes, which were evacuated before and again after warming to 38°. For aerobic incubation 25 cc. were measured into 100 cc. conical flasks. At intervals 20 cc.

of these solutions were measured into 10 cc. water + 10 cc. phosphate (0·2 *M*, p_H 6·46); 5 cc. phenylglyoxal solution were added and samples taken, immediately and after 80 mins.

Fig. 3 shows the results obtained. For the first 4 hours there is no significant difference between aerobic and anaerobic incubation, with or without pancreatin. After this lapse of time the phenylglyoxal-destroying power of the aerobic solutions diminishes rapidly, with no significant difference between the solutions incubated with or without pancreatin. On the other hand anaerobic incubation, in absence of pancreatin, causes a gradual inhibition which continues steadily for at least 8 hours, without the sudden acceleration of the process observed in presence of oxygen. With pancreatin rapid inhibition begins in the fourth or fifth hour, as far as one can judge somewhat later than in the aerobic solutions.

In the first place this experiment shows that both the gradual inactivation of glyoxalase on incubation, and its rapid inactivation after a lag period in presence of pancreatin [Giršavičius, 1933], are independent of the presence of oxygen. The aerobic results are not surprising. Hopkins and Elliott [1931] have found that GSH, in minced liver, is oxidised in presence of oxygen after a preliminary lag period. They explain this by the presence of reducing systems in liver, which have to be depleted by oxidation before the GSH is attacked.

It appears from this experiment that, although both oxygen and pancreatic antiglyoxalase inhibit glyoxalase with superficially similar kinetics, the two effects are independent.

Summary.

1. The p_H-activity curve of glyoxalase, with phenylglyoxal as substrate and phosphate buffer, is investigated by a polarimetric method. The optimum reaction seems to lie in the neighbourhood of p_H 6·4 to 6·5.

2. The effect of GSH concentration on glyoxalase activity (phenylglyoxal destruction) is investigated. About 0·1 % GSH is required for full activation of the enzyme.

3. Moderate access of oxygen causes rapid glyoxalase inactivation, after a lag period. A similar lag period, with subsequent rapid inactivation, characterises antiglyoxalase action. This however is independent of the presence of oxygen.

REFERENCES.

Giršavičius (1931). *Biochem. J.* **25**, 1807.
—— (1932). *Biochem. J.* **26**, 155.
—— (1933). *Biochem. Z.* (In press.)
Hopkins and Elliott (1931). *Proc. Roy. Soc. Lond.* B **109**, 58.
Kuhn and Heckscher (1926). *Z. physiol. Chem.* **160**, 116.
Lohmann (1932). *Biochem. Z.* **254**, 332.

LXXV. THE ACTIVATION OF PAPAIN BY CYANIDE AND OTHER SUBSTANCES. I.

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(*Received March 6th, 1933.*)

Introduction.

It has been known for many years [Vines, 1902; 1903] that papain, the proteolytic enzyme of the fruit juice of *Carica papaya*, the papaw tree of tropical America, is activated by hydrogen cyanide. At later dates hydrogen sulphide [Mendel and Blood, 1910; Willstätter and Grassmann, 1924], cysteine [Grassmann, Dyckerhoff and Schoenebeck, 1930], and reduced glutathione [Grassmann, Schoenebeck and Eibeler, 1931] have been added to the list of activators. The importance attached to the phenomenon has been greatly increased since it has become known that it is not a phenomenon peculiar to papain, but is characteristic of a group of proteolytic enzymes distributed throughout the plant and animal kingdoms. Willstätter, Grassmann and Ambros [1926] showed that the behaviour of papain was paralleled by the enzyme of pineapple (bromelin), and although they believed that the proteases of certain other plants studied—cucumber and pumpkin—displayed a contrary behaviour, Ambros and Harteneck [1929, 2] have since shown that this observation is accounted for by the strong inhibition of the peptidase constituents by cyanide, which, when the method used measures total hydrolysis of peptide linkages, masks the activating effect on the proteinase proper. Grassmann and Dyckerhoff [1928] showed that the proteinase of yeast was activated by cyanide, and finally Waldschmidt-Leitz, Schäffner, Bek and Blum [1930] showed the same for cathepsin, the name given by Willstätter and Bamann [1929] to the proteolytic enzyme of animal tissues such as liver and spleen. The known protein-splitting enzymes appear to fall into three groups of which pepsin, trypsin, and cathepsin or papain are typical; they act in acid, alkaline, and isoelectric solutions of proteins respectively, and are distributed, the first only in specialised parts of the digestive tract of higher animals, the second in all animal phyla but confined to the digestive tract and glands related thereto, and the third apparently throughout the animal and plant kingdom.

Willstätter and Grassmann [1924] propounded a theory of the action of HCN. In the same year in Willstätter's laboratory, Waldschmidt-Leitz [1924] had demonstrated the activator nature of enterokinase, previously held to be itself an enzyme, and it was sought to show an analogy between the activation of papain and that of trypsin. Willstätter and Grassmann suggested that HCN combined with papain to form a new enzyme of extended specificity. Later as a result of the progress in the fractionation of the proteolytic systems, both in yeast and in the animal digestive tract, made by themselves and their collaborators, the theory with regard to both enzymes underwent modification. The

work of Waldschmidt-Leitz and Purr [1929] led them to conclude that the "proteinase" constituent of trypsin is completely inactive in the absence of enterokinase, while the results of Grassmann and Dyckerhoff [1928] and Waldschmidt-Leitz *et al.* [1930] are claimed to show that yeast proteinase and spleen cathepsin respectively are inactive without the presence of HCN or other activator. Papain has not been obtained in an inactive form, but it is assumed by this school to be incompletely separated from a natural activator.

This natural activator in the case of papain was first described by Ambros and Harteneck [1929, 1] under the name of "phytokinase," and in the case of cathepsin by Waldschmidt-Leitz *et al.* [1930] under the name "zookinase." Both these activators have now been identified as reduced glutathione [Waldschmidt-Leitz, Purr and Balls, 1930; Waldschmidt-Leitz and Purr, 1931; Grassmann, Schoenebeck and Eibeler, 1931]. Oxidised glutathione is unable to act as an activator, and so it is possible to link up the activity of the proteolytic mechanism of the cell with an oxidation-reduction system.

Such a connection was suggested by the Munich workers even before the identification of the activators with glutathione [Waldschmidt-Leitz, Bek and Kahn, 1929; Grassmann, Dyckerhoff and Schoenebeck, 1930]. Previously it had been held that the process of autolysis was stimulated by the growing acidity of the tissues, which would bring them to the active range of cathepsin; on this was now superimposed the idea that the enzyme itself is activated through an alteration in the —SH/—S : S— equilibrium in favour of the former, brought about by the diminution in oxygen tension. Scarcity in the supply of oxygen would lead directly to a stimulation of protein metabolism. Waldschmidt-Leitz and Schäffner [1930] went so far as to suggest that tumour growth might be related to increased proteolysis brought about by the high —SH/—S : S— ratio associated with the anaerobic habit of this type of cell.

Acceptance of this view has been by no means general, nor has the idea of the necessity of a ternary system consisting of enzyme, activator (or co-enzyme), and substrate. The most vigorous presentation of an alternative view is that of Krebs [1930]. It was recognised by Grassmann, Dyckerhoff and Schoenebeck [1930] that the chief similarity between the substances serving as activators was that they were all of the group of substances which form compounds with heavy metals. The theory of "detoxication" as an explanation of activation was however rejected by them on several grounds, notably that some of the enzymes had been obtained completely inactive in the absence of the activator. Krebs however maintains that a "detoxication" in the sense of a removal of inhibitors—traces of heavy metals—is the sole explanation. He showed that salts of certain metals—copper, silver, gold, zinc, cadmium and mercury—exert a powerful inhibitory effect in extremely low concentration, an effect which can be nullified by the addition of cyanide, sulphide or cysteine. He claimed that a sufficient concentration of copper and zinc was present, not indeed in the enzyme preparations, but in the commercial gelatin used as substrate, to give a noticeable inhibition unless some metal-binding substance were present. He tried the effect of three further such substances, citrate, pyrophosphate and 8-hydroxyquinoline, and found that they all acted as activators. By removing the last traces of metals from gelatin he claimed to have obtained it in a form in which its hydrolysis was no longer accelerated by HCN. With other substrates which are less contaminated he stated that the phenomenon of activation was not to be observed. Naturally if this view is accepted activation ceases to be a physiological phenomenon at all and any view such as that of Waldschmidt-Leitz falls to the ground.

Other workers have produced evidence which in part supports Krebs's views. Kleinmann, who previously [Kleinmann and Stern, 1930] had reported activation of ox-spleen cathepsin towards caseinogen by HCN, later [Kleinmann and Werr, 1931] stated that with purified cathepsin preparations this was not the case, nor could any activation be discerned in the hydrolysis of the cell proteins, using a nephelometric technique. Abderhalden, Buadze and Geidel [1931] also could find no increase in the rate of tissue autolysis on the addition of glutathione.

Now if HCN acts by combating the effects of heavy metal inhibitors, then for every effect of metal ions on the kinetics of hydrolysis, such as for instance any alteration of the optimum p_H, a correlative effect in the reverse direction should be caused by cyanide. The object of the work to be described was to seek any such effects, *i.e.* to determine whether activation by cyanide *etc.* and inhibition by heavy metal ions show a reciprocal relationship.

The most comprehensive study of the inhibition of an enzyme by metallic ions is Myrbäck's [1926, 1] study of the inactivation of invertase by silver ions. Myrbäck applied the Michaelis and Menten [1913] theory of the enzyme-substrate compound, in the modified form of Euler, Josephson and Myrbäck [1924], according to which all the forms of the enzyme, ionised and unionised, may combine with substrate, but only the compound of the unionised enzyme may break down. This modification is an essential one as it alone explains the independence of the p_H curve on substrate concentration and of the p_S curve on p_H. Already knowing [Euler and Myrbäck, 1922] that the curve relating inhibition to the concentration of silver salt was that typical index of the dissociation compound, the rectangular hyperbola, and also that the inhibition was independent of the substrate concentration, Myrbäck was able to show from a study of the p_H curves in presence of different concentrations of silver salt that the action of silver is explicable on the assumption that silver ions and anions of the enzyme combine to form an only slightly dissociated salt, thus altering the equilibrium between the dissociated and undissociated forms of the enzyme, the readjustment of which leads to a diminution of the concentration of the latter which is the active form. In the equation of the p_H curve, the parameter K_a, the acid dissociation constant of the enzyme, is replaced by $K_a(1 + Ag^+/K_{Ag})$, where K_{Ag} is the dissociation constant of the silver-invertase compound. One limb—the alkaline—of the p_H curve is thus displaced to a parallel position inwards, and the optimum is displaced towards the acid side. The percentage inhibition produced by a given concentration of silver salt becomes progressively greater in passing from the acid to the alkaline side.

Myrbäck's theory was a general one for the inhibition of an enzyme by metal salts, and his work showed that it fitted accurately for the case of invertase, where the substrate is not ionised itself, where one can therefore assume that K_a is the same for the enzyme-substrate compound as for the free enzyme, and where the substrate is unlikely itself to react with the inhibitor. It does not follow that it will hold equally for the case of papain where none of these conditions applies, and the present work shows that it does not, at least in its simple form. It was necessary to make a separate study of the metal-inhibition of this enzyme.

The information already available on the effect of activators on the p_H curve was also insufficient. Frankel [1917], who made a fairly rough study of the optimum p_H, found in cyanide only a broad optimum zone. Willstätter and Grassmann [1924], on the other hand, found a distinct optimum which was the same with and without activator (HCN). Waldschmidt-Leitz *et al.* [1930] also reported the same optimum for spleen cathepsin whether activated by HCN,

H_2S or zookinase (glutathione). This side of the question also required further investigation.

Ambros and Harteneck [1929, 1] showed fairly conclusively that the capacity of papain for activation by cyanide varied considerably with such factors as the state of ripeness of the fruit at the time of picking, as well as with storage of extracts of the enzyme, *etc.* Since it might be expected that metal-inactivation, if a property of the enzyme itself and not of the colloidal material associated with it, would be characteristic and invariant, it was resolved to investigate both phenomena with more than one preparation of enzyme and with extracts subjected to various forms of preliminary treatment.

Methods.

The methods available for the study of proteolysis are far from satisfactory. They may be divided first into two groups, those measuring the disappearance of protein and those measuring the increase of titratable groups. Although the latter methods fail to distinguish between the action of true proteinases and that of peptidases, they are generally considered to be preferable, and nearly all the quantitative studies have been carried out with their aid.

The Willstätter school generally use the method of titration with alcoholic KOH in 90 % alcohol using thymolphthalein as indicator [Willstätter and Waldschmidt-Leitz, 1921]. Although remarkably successful in their hands this method has several difficulties. A control sample of undigested protein has to be titrated and the rapid coagulation of this by the alcohol renders its full titration liable to error. The work of Steudel, Ellinghaus and Gottschalk [1926], who used this method in a study of pepsin, was vitiated, according to Waldschmidt-Leitz and Simons [1926] and Sørensen and Katschioni-Walther [1928], by error in the titration of the control.

Cannon and Muntwyler [1930] used a method of electrometric titration. This served admirably for their purpose but is of course unsuited for kinetic studies where a rapid method suitable for serial experiments is essential. Their titration curves suggested however that results could be obtained by titrating with CO_2-free NaOH in aqueous solution to p_H 10·5. This method on trial however proved unsatisfactory.

The method finally used was that of formaldehyde titration. This method is far from perfect but under suitable conditions gives reproducible results. It will not be out of place to consider a little more deeply the extent of its applicability in this field.

Titration curves of gelatin are published by Cannon and Muntwyler [1930] and by Birch and Harris [1930]. The latter show on the same figure a titration curve in presence of formaldehyde. The formaldehyde titration, that is, the amount of NaOH required to restore the p_H to the value it had before addition of the formaldehyde, varies slightly with the p_H chosen (apart from the variation with the concentration of formaldehyde, which is small if the latter is moderately high, say 4 %), and under no conditions amounts to the equivalent of the $—NH_2$ groups. Some authorities recommend that formaldehyde titrations should be carried out at p_H 8·5, but early in this work the writer found that the values obtained here were appreciably smaller than at p_H 7·5, when titrating papain digests. The difference did not occur in the titration of gelatin or of Savory and Moore peptone. To understand this it was necessary to obtain the titration curves of the digest both in presence of formaldehyde and alone. These curves are shown in Fig. 1, with similar curves for gelatin for comparison. They were obtained

colorimetrically in the following way. A Cole-Onslow comparator was used, and to the tube which was to contain buffer and indicator a known amount of standard KH_2PO_4 or boric acid-KCl mixture (as the case might be) was added, with a suitable indicator. The contents were then brought successively to reactions at intervals of 0·2 p_H by adding standard NaOH, the amount required being read off from the known titration curves given by Sørensen [1909]. The tube containing digest and indicator was titrated to match each tint in turn. The whole curve was obtained in three instalments, using phosphate and bromothymol blue (6·4 to 7·6), borate and cresol red (7·6 to 8·6) and borate and phenolphthalein (8·6 to 9·4).

The dissimilarity in the behaviour towards formaldehyde is made clear.

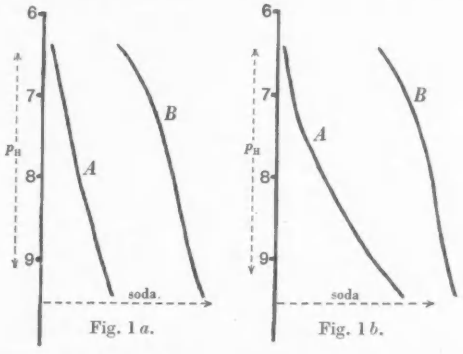

Fig. 1 *a*. Fig. 1 *b*.

Fig. 1. (*a*) Titration curve of gelatin. *A*, alone; *B*, with 4% formaldehyde. (*b*) Titration curve of papain digest of gelatin. *A*, alone; *B*, with 4% formaldehyde.

It is seen that in the case of the digest very few of the amino-groups begin to back-titrate before p_H 7, while most of the —N : CH_2 groups are back-titrated by p_H 7·5. Between these values of p_H the formaldehyde titration is the largest, although it obviously does not represent the equivalents of —NH_2 present. The greatest accuracy in readings is also between these values of p_H, for here the two end-points are of equal degrees of sharpness. The curve shows that at more alkaline p_H, although the end-point in presence of formaldehyde is sharper, the first is less well-defined, while the reverse is true at greater acidity.

In all the experiments described therefore hydrolysis has been measured by formaldehyde titration at p_H 7·2–7·3 in a Cole-Onslow comparator with bromothymol blue as indicator. It has been assumed that this measures a constant fraction of the amino-groups liberated, and this is sufficient in a study that requires only relative velocities of hydrolysis.

Next the problem arises how best to determine these velocities from the data of the titration figures, since the course of hydrolysis is not linear, but some undefinable function of the time.

As substrate gelatin was used throughout. Although the criticism may be made that gelatin is not a complete protein, all other substrates have obvious disadvantages. Caseinogen is insoluble at the p_H-optimum of the enzyme and so are denatured albumin and globulin; native albumins and globulins are frequently attacked very slowly by proteolytic enzymes and may display a latent period before attack commences. As this phenomenon is often ascribed

to a blocking effect of the aromatic residues, the absence of the latter from gelatin is an advantage. The ready availability of gelatin in quantity is also in its favour. Even with gelatin however nothing approaching a linear course of hydrolysis is obtained. This is not to be wondered at, since after the first stage an unknown number of breakdown products, all acting as substrates, is present in the system.

Recourse was had to the principle, outlined by Kuhn [1923], of comparing the times required for a given amount of hydrolysis. This method was checked by experiments in which the only variant was the enzyme concentration. Four samples from each were taken for titration, one immediately after mixing and three at subsequent intervals, and a smooth curve was drawn through the points obtained by plotting the formaldehyde titrations. The time required for each to reach a certain level of hydrolysis was noted and the ratio taken, this being repeated for several levels. When the times were not too widely different the same ratio was maintained throughout and agreed with the ratio of enzyme amounts. If one had very much less enzyme than the other its velocity tended to fall more in the later stages so that the ratio fell gradually. Very much inhibited systems therefore gave the least accurate figures.

The time ratio will obviously only be constant if the kinetics of the two follow the same course. Fortunately the presence of activator or inhibitor does not appear to affect this, and this principle was used in all activation and inhibition experiments. The velocity of the control was usually taken as 100, and the velocity of the activated or inhibited sample equalled the time taken by the control divided by the time taken by the other. When certain other factors are varied, this constancy of the kinetics does not entirely hold. It probably does not when the substrate concentration is altered, though this point did not arise in the present work, and it certainly does not hold strictly when the variant is the p_H. It was always noticed that the departure from linearity was more marked at acid p_H than at alkaline. There is not any serious difference between about p_H 4·4 and 5·2, but on the extreme slopes of the p_H curve it is quite evident. As a consequence a really true p_H curve cannot be constructed. If the experimental times are short, reaction velocities will tend to come out too high on the acid side and too low on the alkaline, if the times are long the reverse will be found. This must be kept in mind in considering the p_H curves, but it does not affect the determination of the optimum.

In the later stages of this work, for comparison's sake various different methods of preparing the enzyme extracts were used, but in the main bulk of the experiments the method of preparing the enzyme solution was as follows. It is the first stage in the method of Kraut and Bauer [1927].

10 g. B.D.H. papain powder were first of all extracted with 50 cc. chloroform, shaking for half an hour and filtering. From the residue the chloroform was removed by brief exposure to a vacuum, and the solid ground up with a small quantity of water, say 25 cc., and then filtered through a very small amount of kieselguhr. The filtrate was diluted about six times and the precipitate formed again filtered after standing.

The substrate concentration unless otherwise stated was 10 cc. of 7 % gelatin in a total volume of 24 cc. The stock 7 % solution sets on standing but is fluid at the temperature of the bath (40°) which it always attained before addition to the mixture of buffer, enzyme, activator or inhibitor and water. The 24 cc. allowed of four titrations and sufficient over for an electrometric determination of the p_H with the quinhydrone electrode. The 5 cc. samples were delivered into 10 cc. water and titrated with standard, roughly $N/10$, CO_2-free NaOH. 2 cc.

of neutralised 40 % formaldehyde was used, making the final concentration of this about 4 %.

Papain digests of gelatin always deposit a precipitate gradually. This may be similar in nature to the plasteins which arise during peptic hydrolysis, although it is a characteristic of pepsin that plasteins are never formed in the hydrolysis of gelatin. The supposed inability of gelatin to form plastein has often been commented upon, and sometimes offered as a further evidence of the incompleteness of gelatin as a protein, this phenomenon being even classed with its inability to act as an antigen.

This analogy seems to break down with the undoubted formation of precipitate in the papain system, which is entitled to the name plastein until more strict definition is given to this term. The production of plastein does not appear to run at all parallel with the extent of hydrolysis, but is controlled in some way by the p_H and salt concentration. On the acid fringe of the p_H curve it is hardly noticeable, but on the alkaline fringe, even where the amount of hydrolysis is very small, heavy plastein deposits occur. Production is less with higher salt concentrations, for instance raising the acetate concentration to five times the ordinary (reaching $M/5$) was found to prevent its formation, or at least its precipitation. This effect has no connection with the activating effect of acetate on the enzyme to be described, for this is complete with a concentration of $M/25$.

Results.

Inhibition by metallic salts at different hydrogen ion and substrate concentrations.

Copper and silver were selected as metals for the study of inhibition, but silver was quickly abandoned for the following reason. When it was used (in concentrations of about 5×10^{-6} M), the ratio of the time-values steadily rose, *i.e.* the inhibitory effect diminished during the course of the experiment. Examining the curve of the hydrolysis course more closely it was found that although it started by showing marked inhibition, after about half an hour it had curved upwards and was running parallel with the control. The most likely explanation seems to be that the silver ions were eluted from the enzyme by the substrate. The power of gelatin to fix silver ions, when on the alkaline side of its isoelectric point, as in these experiments (about p_H 5·2), is well known [Loeb, 1924]. Indeed the remarkable thing is that any inhibition is obtained at all, considering the immense excess of substrate to enzyme. With copper this phenomenon is definitely not observed. The ratio of the time-values remains constant, and with increased time of the inhibited sample an increased falling off of activity, rather than a regeneration, is noticed. Myrbäck [1926, 1] noted that with crude invertase preparations the inhibition was diminished if the enzyme and silver salt stood together for long before addition of substrate; this he attributed to a migration of the silver from combination with the active ionisable group to fixation on the protein carrier. Nothing analogous with this occurs with papain and copper salts, although the enzyme contains protein; very long standing (several days) with the inhibitor somewhat increases the inhibition, perhaps by diminishing the stability.

In view of the ability of the substrate to combine with the inhibitor, it was foreseen that the Myrbäck scheme might not hold good here. There was no field for competition in his experiments and the inhibition by a fixed concentration of metal salt was independent of the substrate concentration. This point was among the first tested in this work and it was found that the inhibition was not so

independent. At higher substrate concentration the inhibition was less (other conditions being kept constant). This is reminiscent of competitive inhibition as encountered with many enzymes, but here, so the writer believes, the competition is not between substrate and inhibitor for the enzyme, but between enzyme and substrate for the inhibitor. Comparative figures for two substrate concentrations are shown in the following table.

Cu^{++} concentration	Reaction velocity as % of velocity in uninhibited control	
	3 % gelatin	4½ % gelatin
$5 \times 10^{-6} M$	70	80
$10^{-5} M$	54	65
$2 \times 10^{-5} M$	32	45
$3 \times 10^{-5} M$	24	33

If this effect is interpreted as the combination of substrate with part of the inhibitor, then this combination will be greater at reactions at which the substrate is more anionised [Loeb, 1924]. A variation of inhibitory capacity with p_H in the contrary sense to that caused by the dissociation of the enzyme in Myrbäck's theory will then ensue, which may be quantitatively great enough to reverse the nett effect, making it one of greater inhibition on the acid side of the optimum, and a shift of the optimum towards alkalinity by the action of the inhibitor. Which effect is greater could only be calculated from a knowledge of the dissociation constant of the enzyme-copper salt and the gelatin-copper salt, and as a number of combining groups are involved in the latter, only an approximate figure could be obtained. The affinity of the enzyme for copper must be exceedingly great or the large excess of substrate would combine with all of it. Experiment shows that the inhibition is in fact greater on the acid side, so that, if this interpretation is correct, the effect of substrate-copper combination is the quantitatively greater one, and this is hardly surprising.

This fact, coupled with those previously discussed as to the limit of interpretation to be put on the p_H curve of a proteolytic enzyme, make it impossible to place this inhibition on the sound mathematical basis upon which Myrbäck was able to put the silver inactivation of invertase, but the empirical facts are excellently suited for comparison with the effect on the p_H curve, *etc.*, of activation by cyanide.

It was decided to confine observations to a single substrate concentration, 10 cc. of 7 % gelatin in 24 cc. total fluid, or nearly 3 %. A standard copper sulphate solution was made up $4{\cdot}8 \times 10^{-4} M$, so that 1 cc. in the 24 cc. was $2 \times 10^{-5} M$. From 0·2 to 2 cc. covered the range of concentrations required.

The observations were first made in acetate buffer and then repeated in phthalate. No difference in the percentage inhibition produced by a given Cu^{++} concentration at a given p_H was observed in the two buffers, but a difference in the absolute velocity was noticed. This was made the subject of further investigation discussed in the next section.

An experiment usually consisted of a series at the same p_H differing only in the inhibitor concentration, one being an uninhibited control. A rough idea of the extent of inhibition to be expected having been found from preliminary experiments, the times at which samples were taken were chosen so that the amount of hydrolysis was roughly equal in each. This minimised the error in the calculation of the time-ratio, ensuring a point on each curve near where the ratios were to be measured. After calculation of the relative velocities as described a curve was drawn relating these to concentration of inhibitor. This

curve, a series of which for differing p_H values is given in Fig. 2, approximated to a rectangular hyperbola. Results were reproducible to an accuracy of 2–3 %, except that from time to time an occasional experiment would give a result right off the curve. The cause of these, common enough in all enzyme work, may

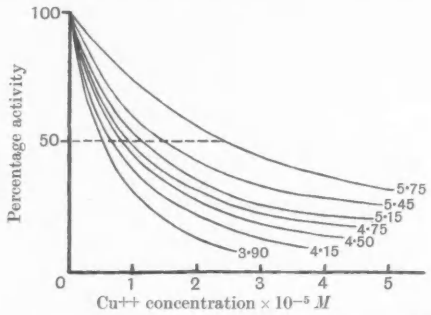

Fig. 2. Inhibition of papain by copper sulphate at various p_H values.

be heterogeneities in the enzyme, or admixture with a trace of unknown inhibitor. The following table gives the concentrations of Cu^{++} giving 50 % inhibition at differing p_H values, read off from the curves in Fig. 2.

p_H	Cu^{++} concentration for 50 % inhibition	p_H	Cu^{++} concentration for 50 % inhibition
3·90	$5{\cdot}6 \times 10^{-6}\,M$	5·15	$1{\cdot}15 \times 10^{-5}\,M$
4·15	$6{\cdot}5 \times 10^{-6}\,M$	5·45	$1{\cdot}45 \times 10^{-5}\,M$
4·50	$8{\cdot}5 \times 10^{-6}\,M$	5·75	$2{\cdot}4 \times 10^{-5}\,M$
4·75	$9{\cdot}3 \times 10^{-6}\,M$		

Activation by acetate.

Before turning to the activating effect of cyanide, the question of the non-agreement of the activity in acetate and phthalate buffers was enquired into. This was found only to occur on the acid side of the optimum. The difference began to be noticeable at p_H 5·0 and grew more marked passing acidwards. At p_H 4·3 the activity in phthalate had fallen to a very small figure and was negligible beyond 4·2. (The p_H was always carefully measured with the quinhydrone electrode.) The activity in acetate was however still quite large, more than six times that in phthalate at 4·3, and extending acidwards to 3·6 or beyond. Drawing the p_H curves for the two buffers showed that both were more or less symmetrical in form and the alkaline limbs identical. Whereas the phthalate had its maximum at p_H 4·9 and then fell to extinction at 4·2, the acetate curve continued to rise (passing from alkaline to acid) until 4·6, whence it fell, parallel with the phthalate curve and 0·6 p_H unit more acidwards (Fig. 3).

This extension or restriction of the zone of p_H activity is similar to the type of salt effect observed with amylase [Myrbäck, 1926, 2], which is so activated or inhibited by many salts, *e.g.* chloride and nitrate, and with fumarase [Mann and Woolf, 1930], activated by phosphate and inhibited by sulphate. The very satisfactory explanation put forward for those cases serves equally here. It is assumed that the enzyme combines with the ions of the salt at some group other than the substrate-binding group and the ionisable groups, and that the p_K of one or both of these ionisable groups, in this case one only, is altered thereby, so

that the fraction of the enzyme remaining in the non-ionised (active) form becomes a different function of the p_H.

To determine which of the two salts was the one combining with the enzyme, or whether both did so, mixtures of the two buffers in varying amounts were used. In this part of the work very special care had to be taken in the measurement of the p_H. For since the gelatin has buffering powers, the p_H of the sample after mixing is not the same as the p_H of the buffer, and this shift is of course different when unequal amounts of buffer are employed. The p_H of each sample was therefore measured separately, and all the activities were plotted on one sheet against p_H. The points were found to lie on families of parallel curves, each corresponding to one concentration of acetate, and entirely independent of the concentration of phthalate (Fig. 3). The salt effect is thus produced by the

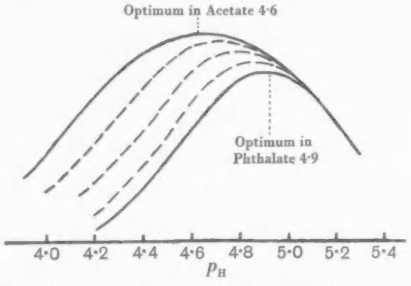

Fig. 3. p_H curves of papain in acetate and phthalate buffers.

acetate alone, the phthalate being passive and apparently not combining with the enzyme. Concentrations of acetate up to $M/25$ produce a shift, after which the curve remains fixed and it is to be taken that all the enzyme is then in the form of enzyme-acetate complex. The affinity of the enzyme for acetate is therefore not very high.

This is the first example of a salt effect of this kind described for a proteolytic enzyme. It is of interest in the repercussion it has on the theory of Northrop [1922], who interpreted the p_H curves of pepsin and trypsin as the titration curves of the substrate, and suggested that the p_H curve of a proteolytic enzyme was determined, not through the ionisation of the enzyme as on the Michaelis theory, but only through the ionisation of the substrate. It is true that the optima for pepsin, trypsin and papain all vary from substrate to substrate, and for papain very nearly coincide with the isoelectric points. The existence of salt effects such as just described makes it certain however that the ionisation of the substrate cannot be the only determinant of the p_H curve since it is not so affected by salts. The truth probably is that both play their part and the p_H curve is something in between the dissociation curves of enzyme and substrate.

	Phthalate		Acetate	
$CuSO_4$ concentration	Actual velocity (arbitrary units)	Relative velocity (standard =100)	Actual velocity (arbitrary units)	Relative velocity (standard =100)
0	66	100	185	100
$6 \times 10^{-6} M$	41	62	109	59
$1{\cdot}4 \times 10^{-5} M$	25	38	70	38

$p_H = 4{\cdot}50$.

It was noted in the last section that the percentage inhibition was the same in phthalate as in acetate. The table on p. 552 gives the actual (arbitrary units) and relative velocities in an inhibition experiment in the two buffers at one p_H.

Activation by citrate.

Citrate is a salt which forms a convenient buffer over the range of activity of papain. It was observed by Krebs to have an activating effect, which he ascribed to its known power of fixing heavy metals, and he maintained that it would replace cyanide as an activator. But many of Willstätter and Grassmann's original results on cyanide activation were obtained in citrate buffer, so that cyanide obviously has an activating effect over and above the maximum effect produced by citrate. This was easily confirmed and it was next shown that addition of citrate to the system already activated by cyanide to the fullest extent attainable by that alone gave a further activation and that of a very large order. Phthalate buffer was present as well to ensure adequate buffering in mixtures containing no citrate, and the p_H was always carefully measured, as the remarks made as to the use of mixed buffers apply again. At p_H 4·4, where the phthalate activity is low even in presence of cyanide, a fivefold activation was produced by adding citrate. The obvious conclusion is that neither cyanide nor citrate replaces the other, but that they activate independently and additively by different mechanisms.

Investigation of the citrate effect shows that it is most likely also a salt effect, since it extends the zone of p_H activity, but it differs from the effect of acetate in that it occurs on both sides of the optimum. On the acid side, at 4·1 the activity is not much less than at the optimum, and the range extends beyond 3·5, while there is a similar extension at the alkaline end. Maximum activation occurs when a concentration of $M/100$ is reached, that is about a quarter the molarity required with acetate, but at least ten times the molarity of cyanide sufficing for full activation.

Since citrate is known to fix heavy metals to some extent, it is to be expected that added copper salt will be partially removed and the percentage inhibition by any given copper concentration reduced to less than that prevailing in acetate or phthalate. This is confirmed by experiment. The fixing is not very efficient or no inhibition would occur at all. Comparative figures for inhibition in citrate and acetate are given in the following table.

	Velocity, relative to velocity of uninhibited sample in same series = 100			
	$p_H = 4·30$		$p_H = 3·90$	
$CuSO_4$ concentration	Citrate	Acetate	Citrate	Acetate
0	100	100	100	100
$8 \times 10^{-6} M$	87	51	—	39
$1·6 \times 10^{-5} M$	77	32	55	16
$3·2 \times 10^{-5} M$	58	14	35	—

In cyanide the inhibition by such small concentrations of copper salt is negligibly small.

Activation by cyanide.

It has already been concluded that cyanide and citrate must exert their activating power in different ways. All the evidence is against the action of cyanide being a salt effect. It does not extend the zone of activity at all, but just causes an increase in the activity over the figure characteristic for the

buffer in use. At reactions at which acetate or citrate produce a salt effect, their addition to the system already fully activated by cyanide causes the appropriate further activation. One must next ask whether the whole effect of cyanide can be attributed to metal-detoxication.

It has been shown here that metal-inactivation causes a shift in the optimum p_H towards alkalinity; Willstätter and Grassmann found no effect on the optimum with activation. They also reported that the activation was quantitatively greatest at the optimum. This statement however may be slightly misleading as it stands; the actual effect is greatest here but according to the experiments of the writer the percentage activation is greatest on the acid side. The following are figures for one enzyme preparation.

p_H	4·0	4·2	4·4	4·6	4·9	5·2	5·4	5·7
Maximum percentage activation	230	230	200	170	170	165	150	135

This result is in support of Krebs's contention, although the results are not so clear-cut. It was this last fact that led to a finding which entirely rules out Krebs's theory as the full and only explanation of the activation. This is that the quantitative effect of cyanide is not constant. The results of Ambros and Harteneck referred to in the introduction were recalled, and the cyanide effect was investigated with different samples of enzyme and with samples of the same enzyme subjected to different treatments and periods of storing.

The preparations tested were:

(1) An aqueous extract of B.D.H. papain, prepared as earlier described. This extract gave a strong Molisch reaction and after hydrolysis with strong HCl gave a powerful reducing sugar reaction.

(2) The same extract digested with saliva and freed from sugars by precipitating the enzyme with 70 % alcohol and redissolving in water; the carbohydrate reaction was almost negative.

(3) The same as (1) autodigested with HCN, aerated to remove this, precipitated and redissolved as in (2).

(4) The same as (1) after treatment in which lead phosphate was precipitated in the solution through addition of ammonium phosphate and then careful and slow addition of just insufficient lead acetate, as recommended by Kraut and Bauer [1927].

(5) An aqueous solution, prepared as before, of another sample of B.D.H. papain, which this time was found to be almost free of carbohydrate.

(6) The same treated as in (2).

(7) The same treated as in (3).

(8) Preparation (5) after six weeks' storage in the ice-chest.

(9) Preparation (5) after 1 month's storage in an incubator at 37°.

The solutions were always stored with toluene. When precipitates settled out they were removed either by filtration or centrifuging. Removal of the precipitate appeared to cause no loss of active material.

It was carefully demonstrated that all these preparations displayed the same behaviour towards copper sulphate. For one substrate and hydrogen ion concentration one copper concentration gave a characteristic constant inhibition. The behaviour towards the substances producing salt effects was also constant. Towards cyanide however they behaved quite differently, though there was a definite regularity about the behaviour. Fresh extracts showed the greatest capacity for activation, a capacity much reduced by storage. This agrees with the spontaneous activation, observed frequently by Grassmann and others,

leading to diminished capacity for further activation. Their explanation of it is that the enzyme, largely at first in the form of the inactive precursor or zymogen, becomes activated on standing by the "phytokinase" or glutathione. Fluctuations in the activity of the preparations occur from day to day, but whether rises are correlated with diminutions in the capacity for activation would be difficult to determine. The climax in this loss of capacity was observed in this work with preparation (9), the one stored in the incubator; within experimental error this material showed no activation at all with the cyanide concentration ($M/1000$) which had proved adequate for limiting activation with other samples. As the substrate in this experiment was commercial powdered gelatin, not subjected to any particular purification, in fact the very material which Krebs maintains is the source of the heavy metal impurity which acts as an inhibitor, it seems as if the magnitude of the Krebs effect must be vanishingly small, at least with the concentrations of cyanide sufficient for the normal phenomenon of activation.

The Willstätter theory, which Krebs stated must be given up, therefore withstands this criticism. It furthermore seems to be the most acceptable theory, for the inconstancy of the cyanide activation rules out most of the forms of activation predictable on the Michaelis enzyme theory. The concept that the cyanide either itself combines with an inactive zymogen to form an active enzyme, or that it liberates reduced glutathione in the cell, which then so acts, in no wise runs counter to the facts. Whether glutathione behaves similarly to cyanide is now being studied but will be reported upon in the future.

Summary.

It is shown that the view of Krebs, that the activation of papain and hence presumably of other proteolytic enzymes of the papain-cathepsin group by cyanide is due solely to the removal of traces of toxic heavy metals, cannot be maintained. The effect of added metal salt—copper sulphate—is characteristic and uniform, a function of the substrate and hydrogen ion concentrations, but invariant from sample to sample. The capacity for activation by cyanide on the other hand is lost by storage in an incubator and partly so by storage in the cold and varies with the mode of preparation of the extract.

The activation by citrate is not, as maintained by Krebs, interchangeable with cyanide, but either salt acts additively to the effect of the other. Citrate activation also extends the zone of p_H-activity in both directions, unlike cyanide, and is shown to be analogous to the activation of salivary amylase by chloride. Acetate displays a similar effect but only on one side of the optimum p_H.

It is a pleasure to thank Sir F. G. Hopkins and Prof. J. B. S. Haldane for their interest in this work.

REFERENCES.

Abderhalden, Buadze and Geidel (1931). *Fermentforsch.* **13**, 147.
Ambros and Harteneck (1929, 1). *Z. physiol. Chem.* **181**, 24.
—— —— (1929, 2). *Z. physiol. Chem.* **184**, 93.
Birch and Harris (1930). *Biochem. J.* **24**, 1080.
Cannon and Muntwyler (1930). *Biochem. J.* **24**, 1012.
Euler, Josephson and Myrbäck (1924). *Z. physiol. Chem.* **134**, 39.
—— and Myrbäck (1922). *Z. physiol. Chem.* **121**, 177.

Frankel (1917). *J. Biol. Chem.* **31**, 201.
Grassmann and Dyckerhoff (1928). *Z. physiol. Chem.* **179**, 11.
—— —— and Schoenebeck (1930). *Z. physiol. Chem.* **186**, 183.
—— Schoenebeck and Eibeler (1931). *Z. physiol. Chem.* **194**, 124.
Kleinmann and Stern (1930). *Biochem. Z.* **222**, 31.
—— and Werr (1931). *Biochem. Z.* **241**, 108.
Kraut and Bauer (1927). *Z. physiol. Chem.* **164**, 10.
Krebs (1930). *Biochem. Z.* **220**, 289.
Kuhn (1923). *Z. physiol. Chem.* **125**, 1.
Loeb (1924). Proteins and the theory of colloidal behaviour. (New York.)
Mann and Woolf (1930). *Biochem. J.* **24**, 427.
Mendel and Blood (1910). *J. Biol. Chem.* **8**, 177.
Michaelis and Menten (1913). *Biochem. Z.* **49**, 333.
Myrbäck (1926, 1). *Z. physiol. Chem.* **158**, 160.
—— (1926, 2). *Z. physiol. Chem.* **159**, 1.
Northrop (1922). *J. Gen. Physiol.* **5**, 263.
Sørensen (1909). *Biochem. Z.* **21**, 131.
—— and Katschioni-Walther (1928). *Z. physiol. Chem.* **174**, 251.
Steudel, Ellinghaus and Gottschalk (1926). *Z. physiol. Chem.* **154**, 21, 198.
Vines (1902). *Ann. Bot.* **16**, 1.
—— (1903). *Ann. Bot.* **17**, 597.
Waldschmidt-Leitz (1924). *Z. physiol. Chem.* **132**, 181.
—— Bek and Kahn (1929). *Naturwiss.* **17**, 85.
—— and Purr (1929). *Ber. deutsch. chem. Ges.* **62**, 2217.
—— —— (1931). *Z. physiol. Chem.* **198**, 260.
—— —— and Balls (1930). *Naturwiss.* **18**, 644.
—— and Schäffner (1930). *Naturwiss.* **18**, 280.
—— —— Bek and Blum (1930). *Z. physiol. Chem.* **188**, 17.
—— and Simons (1926). *Z. physiol. Chem.* **156**, 114.
Willstätter and Bamann (1929). *Z. physiol. Chem.* **180**, 127.
—— and Grassmann (1924). *Z. physiol. Chem.* **138**, 184.
—— —— and Ambros (1926). *Z. physiol. Chem.* **151**, 286.
—— and Waldschmidt-Leitz (1921). *Ber. deutsch. chem. Ges.* **54**, 2988.

LXXVI. CHANGES PRODUCED BY TESTICULAR HORMONE IN NORMAL AND IN CASTRATED RATS.

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(*Received March 7th, 1933.*)

In previous experiments, the testicular hormone was injected only for a short period, generally 7 days and not more than 12 days, and only into castrated rats [Korenchevsky, 1932, 1, 2; Korenchevsky, Schalit and Graetz, 1932; Korenchevsky, Dennison and Schalit, 1932; Korenchevsky, Dennison and Kohn-Speyer, 1932]. During this short period a definite return towards the normal condition was only obtained in the sexual organs and in the thymus. Changes towards a normal state in the fat deposition, adrenals, thyroid and hypophysis were obtained only exceptionally or only to such a slight degree as to be within the limits of physiological variations [see *e.g.* Korenchevsky, 1932, 1, p. 420, Table V; Korenchevsky, Dennison and Kohn-Speyer, 1932, p. 2102, Table VI]. In order to increase these latter changes it seemed necessary that the period of injection should be longer, and that the rats should be used at the end of a longer period after castration, by which time all the changes specific for castrated animals would be more pronounced.

In the present experiments, therefore, the period of injection of 3 weeks was chosen and rats castrated not less than 65 days before the beginning of the experiment were used. In this investigation only young normal rats were used. Experiments on adult normal rats will be published later. The changes were studied not only in the sexual and endocrine organs, but also in the liver, kidneys, spleen and heart. These latter organs increase in weight in rats fed with thyroid preparations. It seemed, therefore, that to study them might be interesting since a possible inter-relationship between the sexual and the thyroid glands has been suggested [Korenchevsky, 1930, 2].

Various doses of the hormone were injected in order to see if, in experiments of longer duration, the apparently direct proportion between the increase of dose and the increase in weight of the sexual organs would be as evident as in the previous short period experiments. The histological changes obtained will be discussed elsewhere.

Technique.

In general, the technique used was the same as that described in previous papers, with one important modification. During the last few years, experience has shown that the prolonged feeding of adult castrated rats with synthetic diet is difficult when a period of $2\frac{1}{2}$ or more months after castration is reached. Whilst normal rats eat a synthetic diet very willingly for any period and at any age, the appetite of many castrated rats for this diet soon progressively declines. The present, as compared with the previous experiments, were not

only of longer duration, but the rats were fed with the diet for some time before the beginning of the experiment. The loss of appetite was consequently more noticeable. Most of the rats stopped growing and many finally began to lose weight slightly. Because of this, during the last 11 days of the experiment, the rats were fed on a mixed diet, which was eaten much better and on which growth was more satisfactory, though still slow.

The mixed diet now in use is more suitable than the synthetic diet for adult castrated rats, but we are still unsatisfied and are trying to find a more suitable diet giving better growth.

The composition of the mixed diet now in use is as follows:

White bread	900 g.
Milk	1100 cc.
Wheat germ	50 g.
Salt mixture[1]	20 g.
Hemp seed	160 g.

To this basal diet were added: 3 times a week rolled oats, 150 g., wheat corn, 150 g.; 4 times a week steamed herrings, 680 g.; and twice a week dried yeast, 120 g. In addition every morning each rat received 2 g. of fresh ox-liver with 3 to 5 drops of cod-liver oil and twice a week 5 g. of carrot. Whole herrings, including head and bones, were used after steaming for 1½ to 2 hours. All the ingredients of the diet were thoroughly mixed and minced into a rough paste. This paste was given in weighed amounts and pressed into earthenware dishes so as to minimise scattering by the rats and to simplify the collection of the residue.

On the following day the uneaten portion was collected and weighed, so that an accurate record was made of the appetite of the rats fed with this paste of natural foodstuffs.

It is here necessary to mention that our various attempts to modify the composition of the synthetic diet, or to use it after steaming, were unsuccessful as regards suitability for feeding adult castrated rats for a long period.

A testicular hormone isolated from urine, dissolved in sesame oil, and prepared by Messrs Schering, Ltd.[2], was used for the injections. Unfortunately it was supplied in two batches. Litter 5 of castrated rats and litters 6, 7 and 8 of normal rats were injected with batch I. This preparation was less purified than that used in our previous experiments [Korenchevsky, Dennison and Kohn-Speyer, 1932]. It was bright yellow in colour and had a pronounced aromatic odour. Litters 1 to 4 of castrated rats and litters 9 to 12 of normal rats were injected with the testicular hormone of batch II. This was a nearly colourless and odourless preparation, probably as well purified as that used in our previous experiments.

Comb-growth assay, which is at present the method mostly used and the one used by Messrs Schering, Ltd., for the standardisation of their preparation, cannot be regarded as giving results which are always comparable [Blyth, Dodds and Gallimore, 1931; see also other references and criticisms on the subject in our previous papers]. Since we have used two quite different batches of possibly different strengths, in the following we shall call the dose marked as "2·5 CU" according to the assay of Messrs Schering's Laboratory "1 D." The other doses used in this paper are designated as parts or multiples

[1] For the composition of salt mixture (No. 1) see Korenchevsky [1922, p. 8].

[2] We wish to express our thanks to Messrs Schering, Ltd., for kindly supplying the preparations.

of this dose. No particular strength therefore is assigned to the dose and we do not assume that one dose of batch I was equal in strength to one dose of batch II, nor is a special general unit assumed. For this reason the averages for each of the batches were made separately, and from the results obtained this arrangement seems to have been justified. Unfortunately the amount of hormone available was not sufficient for all the experiments which had been planned.

The absorption from subcutaneous tissue of testicular hormone dissolved in oil.

The testicular hormone used was dissolved in sesame oil. In our previous experiments [Korenchevsky, Dennison and Schalit, 1932, p. 1312] we noted that on autopsy there was some unabsorbed oil in the subcutaneous tissue at the places where the injections had been more recently made. We therefore suggested that emulsification of the testicular extract was perhaps desirable.

During the present investigation at autopsy special attention was paid to the question of absorption. Not more than 0·1 cc. of oil was injected. We found, as before, some definitely unabsorbed oil at the places at which the injections were made during the last two days. This fact, however, as in the previous experiments, did not influence the accuracy of the comparison of the results obtained. This may have been due, with the doses used in the present and previous experiments (0·1 to 0·15 cc. per dose), to a constant proportional rate of absorption of the oil; or to the fact that the testicular hormone was more readily absorbed from the oil than was the oil itself.

As has been pointed out by Blyth, Dodds and Gallimore [1931], this method of slow absorption of testicular hormone from oil approaches more nearly to the natural process of hormone secretion into the blood stream. We agree with this, and we can also say that our experience has shown that sesame oil can conveniently be used for the assay and for experiments on rats and larger animals. This is important, if the hormone is to be used in oily solution for patients, since the same solvent should be used in the assay as is used in treatment. Otherwise different rates of absorption with resulting different concentrations of the substance in the blood stream may alter the effect of the substance to a considerable degree.

For smaller animals, such as the mouse, oil solutions might prove to be less suitable, since even such a small amount of oil as 0·1 cc. appears large, compared with the small area of subcutaneous tissue of the mouse, especially when the injections have to be performed daily for long periods.

Among our injected animals was one which was about half the weight of its litter-mates and which was emaciated owing to pneumonia affecting 3 lobes of one lung. In the subcutaneous tissue of this animal (which is not included in the Tables) we found a very much larger amount of unabsorbed oil than in any of the other rats. The response of this rat to the injections was also less, which suggests that in animals which are undernourished the absorption of oil and of the hormone from the subcutaneous tissue is less complete than in normal animals.

The desirability of the use by different laboratories of similarly standardised stocks of animals when making quantitative biological tests.

At the present time various preparations are tested and standardised on animals. When choosing the animals to be prepared for a test, the stock, the breeding, environment and diet, the age at which any special preparation of

the animals (*e.g.* operation) is performed, the age at which the test is made, the weights of the animals and the use of controls of the same litter are not always considered. It may be, that not all these data are important for a special test, but, before this can be assumed, special experiments must be carried out in order to separate the important from the unimportant factors.

The standardised animals of the Lister Institute [Smith and Chick, 1926] were used for the experiments. Since, however, the demand for rats sometimes exceeds the supply we considered the use of animals from outside. Before using the animals for our experiments we compared our stock with that of one of the special breeding nurseries. The results are summarised in Table I.

Table I. *Comparison of average weights of normal rats belonging to two different stocks and of average weights of prostate with seminal vesicles and of spleen.*

	Age at weaning days	No. of litters used	Total no. of rats used	Body weight g.	Weight of prostate + seminal vesicles g.		Weight of spleen g.	
					Actual	Per 200 g. body weight	Actual	Per 200 g. body weight
Lister Institute rats:								
Age 21 days	21	10	59	38	—	—	—	—
30 „		5	25	68	—	—	—	—
53–56 days		2	4	199	0·620	0·605	0·532	0·552
65 days		2	4	270	1·075	0·801	0·544	0·436
Nursery's rats:								
Age 21 days	21	2	11	25	—	—	—	—
30 „	30	5	23	42	—	—	—	—
65 „		2	11	174	0·367	0·426	0·849	0·968

Although a comparatively small number of normal animals of the same age were available for the comparison, these showed such essential differences that it was doubtful if assay on the two stocks of animals would give sufficiently close results. As is shown in Table I a difference was noted in the weight and rate of growth of nursery rats which weighed from 34 % to 38 % less than the Lister rats and in the prostate and seminal vesicles, the organs used in the assay of testicular hormone, which weighed 47 % less.

Large spleens (+ 122 %) were also found in two litters of the nursery rats.

As the result of our previous and present observations, we would like to associate ourselves with those who find it desirable to work out standardised conditions of animal stocks, their breeding, their preparation and their keeping during biological assays, in order that results as nearly comparable as possible should be obtained.

It might be possible, perhaps, to overcome some of the difficulties associated with the assay of testicular hormone by the comb-growth test, or of the oestrin test on rats and mice, if, in addition to the conditions of general breeding of the animals, all the data which might influence the results were also taken into consideration, such as the age at which castration is performed, the period after castration, as well as the weight and age of the animals at the beginning of the test.

Castrated rats.

Experiments were performed on 29 rats belonging to 5 litters. The number of days between castration and the commencement of the experiment, the age and the weight of the animals are given in Table II. The age at castration varied

from 23 to 36 days, *i.e.* before the earliest possible date of sexual maturity in our stock (38 days). Injections were started from 65 to 77 days after castration in the first 4 litters and in litter 5 about 1 month later.

The hormone was injected for 21 days in the amount of 1 or 2 D. per day, each dose being given in two daily half doses, one in the morning and a second at about 5.30 p.m. As in previous papers, to economise space, the results are not given for each rat, but only for each group of each litter.

The influence on body weight, food intake and fat deposition.

The data are summarised in Tables II, III and IV. The results of injections of testicular hormone on these functions should be taken with reservation, since during the first part of the experiment the rats were fed on a synthetic diet which they disliked, and only during the last 11 days were they given a mixed diet eaten uniformly and readily by each group. There was, however, one rat in litter 3 which fed badly during the whole experiment, failed to grow and became thin, differing from all the other rats. This rat was probably ill, since the spleen and adrenals were large. No other pathological changes were found on autopsy except a very poor fat deposition. The lungs were normal and the weights of the other organs did not differ from those of the other injected rats. Since, however, this rat differed so much in growth, fat deposition and appetite, these data are not included in the Tables.

As can be seen from Table II, the final weights of all the injected rats are greater than those of the controls. All castrated rats grow slowly, as is shown by the figures for "actual gain in weight," Table III, but the gain in weight of the injected rats was greater than that of the controls.

Table II. *Age and average weight of the castrated rats in each group of each litter.*

No. of litter	No. of rats in litter	Age in days: At castration	Age in days: When injections started	No. of days after castration at beginning of injections	Final weight: Control	Final weight: 1 D.	Final weight: 2 D.
1	4	23	100	77	301	326	—
2	6	29	94	65	319	339	—
3	6	31	108	77	307	334	344
4	5	36	113	77	363	—	392
5	8	28	137	109	303	334	328

Table III. *Influence on gain in weight and food intake of castrated rats.*

Average gain in weight (g.), actual and per 10 g. of actual food intake; and average daily food intake, actual and per 200 g. of body weight, in each group of each litter after the injection of testicular hormone.

No. of litter	Batch of extract	In whole period actual gain (+) or loss (−) in body weight: Controls	1 D.	2 D.	During last 11 days: Change in weight per 10 g. of actual food intake: Controls	1 D.	2 D.	Actual daily food intake: Controls	1 D.	2 D.	Daily food intake per 200 g. body weight: Controls	1 D.	2 D.
1	II	+ 5	+20	—	−0·06	+0·19	—	33	34	—	22	21	—
2	II	+15	+30	—	+0·35	+0·45	—	36	40	—	23	24	—
3	II	+ 9	+35	+29	+0·18	+0·54	+0·39	36	42	40	24	26	24
4	II	+11	—	+35	+0·14	—	+0·44	38	—	41	21	—	21
	Average	**+10**	**+28**	**+32**	**+0·17**	**+0·39**	**+0·42**	**36**	**39**	**41**	**23**	**24**	**23**
5	I	+ 8	+19	+26	−0·11	+0·07	+0·21	18	19	19	12	12	12

Table IV. *Influence on liver, kidneys and retroperitoneal fat of castrated rats.*

The average weights (g.), actual and calculated per 200 g. of body weight, of the organs of the rats in each group of each litter and their percentage change after the injection of testicular hormone.

		Actual weights					Weights per 200 g. of body weight				
			Rats injected with		Percentage change			Rats injected with		Percentage change	
No. of litter	Batch of extract	Control rats	1 D.	2 D.	1 D.	2 D.	Control rats	1 D.	2 D.	1 D.	2 D.
Liver:											
1	II	14·08	16·85	—	+19·7	—	9·34	10·30	—	+10·3	—
2	II	14·09	18·25	—	+29·5	—	8·84	10·78	—	+21·9	—
3	II	13·06	16·95	17·40	+29·8	+33·2	8·57	10·16	11·07	+18·7	+29·1
4	II	15·32	—	20·11	—	+31·3	8·46	—	10·26	—	+21·3
Average	II	**14·14**	**17·35**	**18·76**	**+26·3**	**+32·3**	**8·80**	**10·41**	**10·67**	**+17·0**	**+25·2**
5	I	12·49	16·42	15·94	+31·5	+27·6	8·25	9·81	9·74	+18·9	+18·0
Kidneys:											
1	II	2·049	2·581	—	+26·0	—	1·361	1·583	—	+16·3	—
2	II	2·070	2·368	—	+14·4	—	1·299	1·399	—	+ 7·7	—
3	II	1·960	2·274	2·606	+16·0	+33·0	1·280	1·361	1·676	+ 6·3	+30·9
4	II	2·405	—	3·050	—	+26·8	1·327	—	1·557	—	+17·3
Average	II	**2·121**	**2·408**	**2·828**	**+18·8**	**+29·9**	**1·317**	**1·448**	**1·617**	**+10·1**	**+24·1**
5	I	2·140	2·519	2·562	+17·7	+19·7	1·416	1·506	1·566	+ 6·4	+10·6
Retroperitoneal fat:											
1	II	13·66	15·59	—	+14·1	—	9·08	9·65	—	+ 6·3	—
2	II	13·90	14·80	—	+ 6·5	—	8·71	8·73	—	+ 0·2	—
3	II	13·62	14·55	16·53	+ 6·8	+21·4	8·84	8·73	9·62	− 1·2	+ 8·8
4	II	17·82	—	18·76	—	+ 5·3	9·84	—	9·58	—	− 2·6
Average	II	**14·75**	**14·98**	**17·65**	**+ 9·1**	**+13·4**	**9·12**	**9·04**	**9·60**	**+ 1·8**	**+ 3·1**
5	I	11·16	16·38	15·03	+46·7	+34·7	7·36	9·78	9·09	+32·9	+23·5

The actual food intake of the injected rats was also slightly greater than that of the controls, but when calculated for the same unit of body weight it was in most cases nearly the same (Table III). It is noteworthy that for the same unit of food (actual figures) ingested the growth of the injected rats was better than that of the controls (Table III, Columns 6, 7 and 8). These results are similar to those obtained when studying the influence of vitamin A on rats kept on a diet deficient in this vitamin [Sampson and Korenchevsky, 1932]. The data given above indicate that testicular hormone not only improves the appetite and growth of castrated rats, but probably takes part in the anabolic processes of tissue building. However, for the confirmation of this latter, *i.e.* the anabolic action of the hormone, it will be necessary to perform further experiments using paired feeding of the animals.

In most litters the weights of the retroperitoneal fat of the injected rats (Table IV) did not differ significantly from those of the controls. A larger increase of 32·9 % (1 dose) and 23·5 % (2 doses injected) per 200 g. of rat weight was obtained in one litter only (litter 5).

It is, therefore, probable that the larger gain in weight of the injected rats as compared with the uninjected controls was due to the more efficient building of active tissues and not exclusively to an increase in fat deposition.

The influence on liver and kidneys.

As can be seen from Table IV there was, on the average, an increase in the weight of both the liver and kidneys of injected animals as compared with the controls both in the actual figures and when calculated per 200 g. body weight.

In most cases a double dose of the hormone had a greater effect. A more definite quantitative relation between the increase in dose and increased effect was not evident, though this relation is clearer when considering the weights calculated per 200 g. of body weight. Even after the injection of the larger dose the average increase in weight of the liver, calculated per 200 g. of body weight, did not exceed 29·1 %, the average for batch I being 18 % and for batch II 25·2 %. The corresponding average increases in the weights of the kidneys were 10·6 and 24·1 % (maximum 30·9 %).

These increases are not large, but they are significant since in our rats in most litters the weights of the livers (as was the case with the other organs) of the rats in each group were similar, in some cases strikingly so.

For example, in litter 4 the weights of the livers per 200 g. of body weight were in the control rats 8·51 and 8·40 g. and in three injected rats 10·29, 10·33 and 10·17 g. The corresponding figures for the kidneys were 1·304 and 1·349 g. (controls); 1·508, 1·615 and 1·547 g. (injected rats).

Again, in litter 5, the weights of the livers of the control rats were 8·39 and 8·11 g.; of the injected rats (1 dose), 10·06, 9·12 and 10·24 g.; the kidneys of the control rats were 1·478 and 1·354 g. and of the injected rats, 1·350, 1·553 and 1·614 g.

Taking into consideration the small degree of variation in the weights of the organs in each group of most of the litters we feel justified in concluding that testicular hormone increased the weight, not only of the sexual organs (see previous papers and following Tables), but of the liver and kidneys. This stimulating effect of testicular hormone on these organs corroborates its anabolic properties which were discussed on the basis of other data in a previous paragraph.

The influence on prostate with seminal vesicles. (Table V.)

In a previous paper [Korenchevsky, Dennison and Kohn-Speyer, 1932] it was shown (i) that for doses of from 1 to 7·5 CU there was an apparent direct proportion between the increase in the dose and the average increase in the

Table V. *Influence on prostate with seminal vesicles and penis of castrated rats.*

The average weight (mg.), actual and calculated per 200 g. of body weight, of the organs of the rats in each group of each litter and their percentage increase after the injection of testicular hormone.

		Actual weights					Weights per 200 g. of body weight				
			Rats injected with		Percentage increase			Rats injected with		Percentage increase	
No. of litter	Batch of extract	Control rats	1 D.	2 D.	1 D.	2 D.	Control rats	1 D.	2 D.	1 D.	2 D.
Prostate with seminal vesicles:											
1	II	82	328	—	300	—	55	202	—	267	—
2	II	86	327	—	280	—	54	193	—	258	—
3	II	73	317	569	334	679	48	190	338	296	604
4	II	103	—	674	—	554	57	—	345	—	505
Average	II	**86**	**324**	**622**	**305**	**617**	**54**	**195**	**342**	**274**	**555**
5	I	71	480	770	576	985	47	290	470	515	900
Penis:											
1	II	105	236	—	125	—	70	145	—	107	—
2	II	107	211	—	97	—	67	125	—	87·5	—
3	II	89	218	246	145	176	58	131	158	126	172
4	II	122	—	326	—	167	67	—	165	—	146
Average	II	**106**	**222**	**286**	**122**	**172**	**66**	**134**	**162**	**107**	**159**
5	I	84	225	240	167·8	185·6	55	135	146	145·4	165·4

weight of prostate with seminal vesicles, and (ii) that, when the preparation used in those experiments was injected for 7 days, 2·5 CU increased the weight of prostate with seminal vesicles by about 106 % and 5 CU gave an increase of 191 %.

From the last two columns of Table V it is seen that batch II gave an average increase in the weight of prostate with seminal vesicles (per 200 g. of body weight) of 274 % with a single dose and 555 % with a double dose. In this experiment with batch II, therefore, we have, as in the previous experiments, a strikingly close direct proportional relationship between the increase in the dose and increase in the size of the gland, for by multiplying 274 % by two the expected increase is found to be 548 %—the actual result obtained being 555 %.

In considering batch I (see Table V) it should be noted, as was stated in the paragraph on technique, that the appearance of this batch was entirely different from that of the other batches, and it proved in our experiments to be about double the strength of batch II, since (per 200 g. of body weight) the increase produced by a single dose was 515 % and by a double dose 900 %. The average weights of the rats of the different litters were mostly similar (see Table II). We have to allow for the fact that (i) the injections of batch I were made on one litter only (and not on at least three as we consider necessary for accurate assay), and (ii) that the litter was a month older and had been castrated a month longer when the injections were started. Such a striking difference, however, in the influence of the two preparations would still seem to be only explained by the suggestion that the comb-growth standardisation of the two different preparations did not give comparable results, as has been found in other instances [Blyth, Dodds and Gallimore, 1931; Kabak, 1931; Champy, 1931].

With regard to the correlation between increase of dose and increase of effect in this litter (No. 5), injections of a single dose gave an increase in the weight of prostate with seminal vesicles of 243 mg. The increase for the double dose should, therefore, be 486 mg. The experimental result gave only 423 mg., about 13 % less than the expected result.

In our previous paper [Korenchevsky, Dennison and Kohn-Speyer, 1932, p. 2101, Table V, col. 5] the maximum deviation of the average increase of prostate with seminal vesicles, between the different litters, injected with the same preparation, did not exceed 12·3 %. The deviation in the present experiment of 13 % is, therefore, quite close to the maximum deviation obtained in the short duration experiments. It must be remembered, however, that batch I was tested on only one litter.

From the figures both of the previous experiments and of the present experiments it is seen that there are often small deviations from the average in the degree of response of rats of different litters injected with the same dose. For example, an injection of a single dose gave an increase in weight of prostate with seminal vesicles (per 200 g. of body weight) of 274 % (Table V), obtained by averaging the increases in the three litters, 1, 2 and 3, these being 267, 258 and 296 % respectively. Thus the deviations from the average were − 2·6, − 5·8 and + 8·0 %. In the case of the double dose (Table V, last column) the deviation from the average of 555 % was ± 8·8 %. The weights of the rats being similar, the actual figures give similar results (Table V).

Therefore, as was found for injections during 7 days, daily injections during 21 days of the same preparation into litters of rats castrated before sexual maturity and not differing greatly in age and weight gave increases in the weight

of prostate with seminal vesicles which are strikingly close, the maximum deviation from the average figure being not more than 9 %.

The double dose, as compared with the single dose, gave in one case (assay on litters 1 to 4) an increase which was practically the same as the expected increase, calculated from the simple dose by direct proportion (about 1·3 % larger). In the other case, litter 5 (one litter only), the increase was 13 % less than that expected. Therefore, the direct proportional relationship between the dose and the effect was, as in the 7-day experiments, again apparent in the case of daily injections for 21 days, the dose injected not exceeding 5 CU.

The nature of this relation will probably change with injections for a longer period or with larger doses, since with the doses injected only a small part of the curve giving the relation between the dose and the effect was represented.

The influence on penis. (Table V.)

As was shown in the previous paper [Korenchevsky, Dennison and Kohn-Speyer, 1932], in experiments of 7 days' duration there was an approximately direct proportional relation between the varying doses and the effect on the penis, though this correlation was much less regular than in the case of prostate with seminal vesicles, nor was the response so sensitive. A dose of 2·5 CU (assayed by Schering's Laboratories to be contained in 1 D. as designated in the present paper) injected daily for 7 days gave an increase in the weight of penis (per 200 g. of body weight) of about 39 %, whilst the double dose gave an increase of about 68 %. Injections for 21 days gave an average increase for a single dose of 107 % (batch II) and 145 % (batch I).

The double doses, however (Table V), did not give double the effect on the penis, but much less. These changes in weight of the penis, therefore, show that prolonging the period of injection to 21 days was followed by the breakdown of the approximate direct proportional relationship between the dose and its effect on the penis. The influence on the penis of batch I seems to be greater than that of batch II. This was, however, evident only in the case of the single doses, there being little difference in the effects of the double doses (Table V), batch II on the average increasing the weight of the penis (per 200 g. of body weight) in litters 3 and 4 by 172 % and 146 % respectively, and batch I (litter 5) by 165 %. The change in weight of prostate with seminal vesicles suggests that the strength of batch I is much greater than that of batch II. The results on the penis agree with this, namely, that the double dose of the same extract and the single dose of the stronger extract were unable to increase the effect on the penis in direct proportion beyond a definite limit. There was, therefore, a limit beyond which increased dosage and increased period of injections had a much less stimulating effect on the growth of the penis. There was thus a definite difference in this respect between the response of the prostate with seminal vesicles and of the penis, within the conditions of dosage and period of injection of our experiments. More numerous experiments, however, are necessary before a definite conclusion can be drawn as to the character of the curve relating the different doses and the effect of testicular hormone on the penis.

The influence on thymus. (Table VI.)

In the present investigation, as in all our previous experiments, injection of testicular hormone hastened involution of the thymus. The increased period of injection was, on the average, followed by a greater decrease in the weight

of the thymus. Thus, after 7 days of injection with doses similar to those used in the present investigation, the decrease in weight of the thymus (per 200 g. of body weight) varied from 4·4 to 27·5 % [Korenchevsky, Dennison and Kohn-Speyer, 1932, p. 2100, Table IV]. In the present experiments after 21 days of injection the average decrease varied from 21·1 to 36·5 % for the single dose and from 28·2 to 47·1 % for the double dose (Table VI, last two columns). Although on the average there was an increased involution with the increased dose, it was of so irregular a nature that it cannot be estimated on the basis of such a small number of figures.

Table VI. *Influence on thymus, adrenals, thyroid and hypophysis of castrated rats.*

The average weights (mg.), actual and per 200 g. of body weight, of the glands of the rats in each group of each litter and their percentage change after the injection of testicular hormone.

		Actual weights					Weights per 200 g. of body weight				
			Rats injected with		Percentage change			Rats injected with		Percentage change	
No. of litter	Batch of extract	Control rats	1 D.	2 D.	1 D.	2 D.	Control rats	1 D.	2 D.	1 D.	2 D.
Thymus:											
1	II	391	322	—	−17·6	—	259	196	—	−24·3	—
2	II	546	368	—	−32·6	—	342	217	—	−36·5	—
3	II	308	240	192	−22·1	−37·7	202	144	122	−28·7	−39·6
4	II	439	—	250	—	−43·1	242	—	127	—	−47·1
Average	II	**421**	**310**	**221**	**−24·1**	**−40·4**	**261**	**186**	**125**	**−29·8**	**−43·4**
5	I	214	190	167	−11·2	−22·0	142	112	102	−21·1	−28·2
Adrenals:											
1	II	66·4	56·8	—	−14·5	—	44·1	35·0	—	−20·6	—
2	II	94·0	69·9	—	−25·6	—	59·2	41·4	—	−30·1	—
3	II	79·3	49·5	77·6	−37·6	−2·1	52·4	29·7	49·5	−43·3	−5·5
4	II	61·2	—	48·5	—	−20·7	33·8	—	24·8	—	−26·0
Average	II	**75·2**	**58·7**	**63·1**	**−25·9**	**−11·4**	**47·4**	**35·4**	**37·2**	**−31·3**	**−15·8**
5	I	69·8	48·3	46·5	−30·8	−33·4	46·2	28·9	28·4	−37·4	−38·5
Thyroid:											
1	II	24·7	31·7	—	+28·3	—	16·4	19·6	—	+19·5	—
2	II	27·9	33·1	—	+18·6	—	17·5	19·5	—	+11·4	—
3	II	28·4	34·8	30·4	+22·5	+7·0	18·5	20·9	19·4	+13·0	+4·9
4	II	34·4	—	36·1	—	+4·9	19·0	—	18·4	—	−3·2
Average	II	**28·9**	**33·2**	**33·3**	**+23·1**	**+6·0**	**17·9**	**20·0**	**18·9**	**+14·6**	**+0·9**
5	I	26·4	34·8	31·8	+31·8	+20·4	17·3	21·0	19·4	+21·4	+12·1
Hypophysis:											
1	II	12·6	14·0	—	+11·1	—	8·4	8·7	—	+3·6	—
2	II	13·2	13·1	—	−0·8	—	8·3	7·7	—	−7·2	—
3	II	13·7	13·9	15·3	+1·4	+11·7	9·0	8·3	9·9	−7·8	+10·0
4	II	13·2	—	14·7	—	+11·4	7·3	—	7·5	—	+2·7
Average	II	**13·2**	**13·7**	**15·0**	**+3·9**	**+11·6**	**8·3**	**8·2**	**8·7**	**−3·8**	**+6·4**
5	I	13·0	12·4	12·1	−4·6	−6·9	8·7	7·4	7·5	−14·9	−13·8

As compared with the figures calculated per 200 g. of body weight, the percentage decreases in the actual weights of the thymus (Table VI, columns 6 and 7) show a much closer relation between the increase of the dose and increased speed of involution: thus with batch II the average decrease is 24·1 % for the single dose and 40·4 % for the double dose; the figures for batch I being 11·2 % and 22 % respectively, *i.e.* the proportion appeared to be direct.

It is possible that, if our rats had been fed with a more suitable mixed diet during the whole period of the experiment, the discrepancy between the actual decrease in weight of the thymus and the weight calculated per 200 g. of body weight would have been less.

The influence on adrenals. (Table VI.)

The adrenals, which hypertrophy in castrated animals [Korenchevsky, 1930, 2; 1932, 2], after injection on the average decreased both in actual weight and when calculated per 200 g. of body weight. No definite difference between the effects of the single and double doses was obtained. The negligible decrease in one group of rats (litter 3) receiving a double dose was probably due to the influence of infection on the adrenals, as has already been mentioned in the paragraph on the influence on body weight, *etc.* The typical influence of the double dose of testicular hormone is demonstrated by the decrease in weight of the adrenals of litters 4 and 5, in which it was similar to those obtained by the injection of the single dose. When the injection period was only 7 days [Korenchevsky, Dennison and Kohn-Speyer, 1932, p. 2102, Table VI] the largest average changes (per 200 g. of body weight) varied between 10 % and 16 %. In the present experiments the average decrease of the adrenals (per 200 g. of body weight) varied in different litters between 20·6 % and 43·3 % (excluding group 2 D., litter 3 for the reason mentioned above).

It follows, therefore, both from our previous and our present experiments, that there was no definite increase in the changes in the adrenals on increasing the dose; and that the changes were increased when the period of injection was increased. It is tempting to conclude that the increased duration of the injection has a greater effect than the increased dose. The available data, however, do not warrant so definite a conclusion, since, as compared with the previous experiments of short duration, the rats used for the present investigation had been castrated for a longer period, so that the changes due to castration were more pronounced, and the effect of the testicular hormone might be more evident than in rats injected sooner after castration.

Of the rats injected for 7 days, only one litter (No. 8) had been castrated 75 days before the injections were started, and in this case the average decrease in weight of the adrenals was only 14·5 % [Korenchevsky, Dennison and Kohn-Speyer, 1932, p. 2105, Table VII]. This result supports the suggestion made above, but as one experiment is insufficient it would be necessary to make a special investigation on a larger number of rats before drawing a definite conclusion.

The influence on thyroid. (Table VI.)

The results are given in Table VI. The weight and size of the thyroid glands decrease in castrated rats [Korenchevsky, 1930, 2]. It was found, in previous experiments, that after short periods of injection of from 7 to 12 days there was little or no increase in weight of the thyroid, the increase not exceeding 13·4 % (per 200 g. of body weight). An increase of 13·4 % was also obtained in the rats of litter 8, which litter was discussed in the preceding paragraph on adrenals, and described in the previous paper. This litter can be compared from the point of view of age and number of days since castration with those described in this paper.

In the present experiments the increase in weight of the thyroids after the injection of the single dose occurred in most rats, being on the average only slightly greater than in the previous experiments, and was negligible after the

double dose. In view of such slight and complicated results more numerous experiments are necessary to decide whether this difference in the effects of the small and large dose was accidental or indicates the presence of some thyroid-depressing, as well as thyroid-stimulating, substances in the testicular extract prepared from urine.

The influence on hypophysis, spleen and heart. (Table VI.)

The hypophysis of castrated rats hypertrophies. Korenchevsky [1930, 2] has found that the average weight of the hypophysis in normal rats was 6·1 to 6·6 mg. per 200 g. of body weight and in castrated rats was about 8·9 mg. In the present experiments (Table VI) the average weight in 5 litters was 8·3 mg., which weight is very close to that given above of 8·9 mg. and also to the figures obtained in another investigation [Korenchevsky, 1932, 2, p. 1302, Table II], in which the weight in rats killed from 30 to 182 days after castration was 8·6 to 9·0 mg.

In the present experiments of 21 days' duration, as in the previous experiments of short duration, the changes in the hypophysis were slight and irregular. It is, therefore, possible to conclude that the testicular preparation isolated from urine produced no definite change towards the normal condition in the weight of this gland. There were no definite changes in the weight of the spleen or of the heart after injection and the figures for these organs are not included in this paper in order to economise space.

NORMAL RATS.

The experiments were performed on 33 rats belonging to 7 litters; the ages and weights of the rats are given in Table VII. The rats were sexually immature at the beginning of the experiment, being between 30 and 35 days old. The same two testicular preparations as were used for the castrated rats, batch I (litters 6 to 8) and batch II (litters 9 to 12), were injected daily for 21 days, three different doses being used, 0·25, 0·5 and 2·0 D. Young rats were used in order to study the effect of the testicular hormone on growth and sexual development, as well as on the weights of the different organs.

The influence on the weight of the rats, food intake and fat deposition.

The data are summarised in Tables VII, VIII and XI. Whilst in castrated rats only the retroperitoneal fat was weighed, in normal rats the weight of the retroperitoneal *plus* testicular fat was estimated. There was no constant or evident difference, in the final weight, gain in weight and food intake between the control and injected rats. It should, however, be noted that in spite of a decrease in the appetite in the injected rats of litters 9, 12 and more particularly

Table VII. *Age and average weight of the normal rats in each group of each litter.*

No. of litter	No. of rats in litter	Initial age (days)	Final weight of rats			
			Controls	0·25 D.	0·5 D.	2 D.
6	6	30	180	175	181	—
7	5	35 (about)	215	—	225	—
8	5	32	150	—	—	144
9	3	35	247	—	250	—
10	4	30	193	—	215	—
11	4	30	203	—	213	—
12	6	30	189	—	—	184

Table VIII. *Influence on gain in weight and food intake of normal rats.*

Average gain in weight, actual and per 10 g. of actual food intake; food intake, actual and per 200 g. of body weight of the rats, in each group of each litter, after the injection of testicular hormone.

No. of litter	Control rats	Rats injected with 0·25 D.	0·5 D.	2 D.	Control rats	Rats injected with 0·25 D.	0·5 D.	2 D.
Actual gain in weight:					Actual food intake:			
6	112	106	109	—	20	19	20	—
7	124	—	136	—	23	—	25	—
8	95	—	—	81	19	—	—	16
9	140	—	136	—	26	—	25	—
10	120	—	138	—	22	—	25	—
11	126	—	133	—	22	—	23	—
12	117	—	—	114	21	—	—	20
Gain in weight per 10 g. actual food intake:					Food intake per 200 g. body weight:			
6	2·7	2·7	2·6	—	32	31	32	—
7	2·6	—	2·6	—	30	—	32	—
8	2·4	—	—	2·4	37	—	—	31
9	2·6	—	2·6	—	29	—	27	—
10	2·6	—	2·6	—	33	—	32	—
11	2·7	—	2·8	—	31	—	31	—
12	2·7	—	—	2·7	32	—	—	31

Table IX. *Influence on testes of normal rats.*

The average weights (g.), actual and calculated per 200 g. of body weight, of testes of normal rats in each group of each litter and their percentage change after the injection of testicular hormone.

No. of litter	Batch of extract	Control rats	Rats injected with 0·25 D.	0·5 D.	2 D.	Percentage change 0·25 D.	0·5 D.	2 D.
A. Actual weights:								
6	I	1·837	1·660	1·468	—	−9·6	−20·1	—
7	I	2·238	—	2·023	—	—	− 9·6	—
8	I	1·599	—	—	0·648	—	—	−59·4
Average	I	**1·891**	**1·660**	**1·746**	**0·648**	**−9·6**	**−14·9**	**−59·4**
9	II	2·268	—	2·436	—	—	+ 7·4	—
10	II	1·976	—	1·890	—	—	− 4·4	—
11	II	1·817	—	1·793	—	—	− 1·3	—
12	II	1·739	—	—	1·132	—	—	−34·9
Average	II	**1·950**	—	**2·040**	**1·132**	—	**− 0·6**	**−34·9**
B. Weights per 200 g. of body weight:								
6	I	2·052	1·897	1·610	—	−7·6	−20·6	—
7	I	2·085	—	1·794	—	—	−13·9	—
8	I	2·135	—	—	0·893	—	—	−58·2
Average	I	**2·091**	**1·897**	**1·702**	**0·893**	**−7·6**	**−17·3**	**−58·2**
9	II	1·842	—	1·948	—	—	+ 5·8	—
10	II	2·050	—	1·758	—	—	−14·2	—
11	II	1·794	—	1·688	—	—	− 5·9	—
12	II	1·851	—	—	1·230	—	—	−33·5
Average	II	**1·884**	—	**1·798**	**1·230**	—	**− 4·8**	**−33·5**

of litter 8, as compared with the controls, the gain in weight per 10 g. of food intake (Table VIII) was the same in both groups.

The decreased appetite in these three litters was accompanied by a decreased deposition of fat. The changes are small and the number of experiments few, it is, therefore, only possible to suggest that testicular hormone does not prevent the building of new tissue in normal rats, but that large doses of the hormone probably affect the stores of fat. In general the influence of the testicular hormone

isolated from urine, especially in the large doses used, unlike the favourable effect on castrated rats, did not improve the weight, appetite or fat deposition of the normal rats.

The influence on testes.

It is clear from Table IX that in general the testicular hormone had a depressing influence on the testes. This is even more evident in the figures calculated per 200 g. of body weight. Whilst small doses produced only a slight effect, the changes in two litters (Nos. 9 and 11) being within the physiological variations, large doses quite definitely decreased the weight of the testes by 33·5 % and 58·2 %. It must be admitted that 5 CU per day per 125 g. of body weight (the average weight for the whole period of injection) for a normal rat having normal testes is a very large dose of the hormone. This would be equivalent for a man of 60 kg. to about 2400 CU per day.

Taking this into consideration doses of 0·25 D. and 0·5 D. (though still comparatively large) were injected into the rats, when the depressing effect was found to be much less. Although large injections are not likely to be repeated in the treatment of patients, a study of the effects of these doses is important in order to show the effect of hyper-secretion and the possible danger of overdosage, especially in the case of young males with normal testes.

Preliminary histological investigation has shown that in the testes of the injected rats normal development of the seminiferous tissue was delayed.

The influence on prostate with seminal vesicles and on penis.

As with castrated rats, changes in the weights of the prostate with seminal vesicles (Table X) and of the penis (Table XI) occurred simultaneously in the normal rats and were similar in character in each group of rats, being less pronounced in the penis than in the prostate with seminal vesicles.

Table X. *Influence on prostate with seminal vesicles of normal rats.*

The average weights (mg.), actual and calculated per 200 g. of body weight, of the prostate and seminal vesicles of the normal rats in each group of each litter and their percentage change after the injection of testicular hormone.

No. of litter	Batch of extract	Control rats	Rats injected with 0·25 D.	Rats injected with 0·5 D.	Rats injected with 2 D.	Percentage change 0·25 D.	Percentage change 0·5 D.	Percentage change 2 D.
A. Actual weights:								
6	I	565	459	399	—	−18·8	−29·4	—
7	I	648	—	503	—	—	−22·4	—
8	I	401	—	—	629	—	—	+56·9
Average	I	**538**	**459**	**451**	**629**	**−18·8**	**−25·9**	**+56·9**
9	II	839	—	675	—	—	−19·5	—
10	II	426	—	512	—	—	+20·2	—
11	II	478	—	434	—	—	− 9·2	—
12	II	534	—	603	—	—	—	+12·9
Average	II	**569**	—	**556**	—	—	**− 2·8**	**+12·9**
B. Weights per 200 g. body weight:								
6	I	628	524	439	—	−16·6	−30·1	—
7	I	604	—	447	—	—	−26·0	—
8	I	537	—	—	873	—	—	+62·5
Average	I	**590**	**524**	**443**	**873**	**−16·6**	**−28·1**	**+62·5**
9	II	672	—	540	—	—	−19·6	—
10	II	442	—	478	—	—	+ 8·2	—
11	II	470	—	404	—	—	−14·0	—
12	II	559	—	658	—	—	—	+17·7
Average	II	**536**	—	**520**	—	—	**− 8·5**	**+17·7**

Table XI. *Influence on penis, kidneys and intra-abdominal fat of normal rats.*

Average percentage changes in weight (actual and per 200 g. of body weight) of the organs in each group of each litter of the rats injected with testicular hormone as compared with those of control rats.

No. of litter	Batch of extract	Penis			Kidneys			Intra-abdominal fat		
		0·25 D.	0·5 D.	2 D.	0·25 D.	0·5 D.	2 D.	0·25 D.	0·5 D.	2 D.
A. Actual weights:										
6	I	−6·1	− 8·9	—	+5·2	+10·8	—	−6·1	+16·5	—
7	I	—	−20·7	—	—	+10·1	—	—	+12·7	—
8	I	—	—	+17·1	—	—	+15·2	—	—	−53·5
Average	I	**−6·1**	**−14·8**	**+17·1**	**+5·2**	**+10·5**	**+15·2**	**−6·1**	**+14·6**	**−53·5**
9	II	—	− 4·5	—	—	+ 8·3	—	—	−12·9	—
10	II	—	+11·8	—	—	+19·8	—	—	+15·4	—
11	II	—	−10·4	—	—	+12·1	—	—	+31·5	—
12	II	—	—	+ 1·7	—	—	+17·6	—	—	−23·6
Average	II	—	**− 1·0**	**+ 1·7**	—	**+13·4**	**+17·6**	—	**+11·3**	**−23·6**
B. Weights per 200 g. of body weight:										
6	I	−4·0	−10·0	—	+8·0	+ 9·8	—	−2·3	+17·8	—
7	I	—	−24·2	—	—	+ 5·2	—	—	+ 9·5	—
8	I	—	—	+22·7	—	—	+17·9	—	—	−50·3
Average	I	**−4·0**	**−17·1**	**+22·7**	**+8·0**	**+ 7·5**	**+17·9**	**−2·3**	**+13·7**	**−50·3**
9	II	—	− 5·0	—	—	+ 6·8	—	—	−14·1	—
10	II	—	+ 0·5	—	—	+ 7·7	—	—	+ 4·0	—
11	II	—	−14·2	—	—	+ 7·3	—	—	+25·0	—
12	II	—	—	+ 4·0	—	—	+20·8	—	—	−21·4
Average	II	—	**− 6·2**	**+ 4·0**	—	**+ 7·3**	**+20·8**	—	**+ 5·1**	**−21·4**

The weight of the prostate with seminal vesicles of the control rats was much below the maximum development, which under normal conditions is reached at a later age, when these glands may be more than 100 % heavier than in the rats of the experiment [see Korenchevsky, 1930, 2].

A priori it would be expected that injections of testicular hormone would stimulate the growth of these organs.

This effect was quite definitely produced by large doses of batch I in the three rats of litter 8, in which the average increase in weight (per 200 g. of body weight) was 62·5 % for the prostate with seminal vesicles and 22·7 % for the penis. Larger doses of batch II produced the much smaller increases of 17·7 % and 4 % respectively (Tables X and XI). This difference between the actions of these two extracts is in agreement with our results on castrated rats, that batch II was weaker than batch I.

With small doses, however, the unexpected result was obtained of slightly delaying the development of the sexual organs of most of the injected rats.

Although the decrease in weight of the prostate with seminal vesicles was only small, not exceeding 30 %, this action on the average was shown, with the exception of litter 10, in all the other litters.

If this discrepancy between the influence of large and small doses is not accidental and not within the limit of physiological fluctuations, it requires further special experiments, since more than one explanation of this fact could be given from the present evidence.

The influence on the kidneys.

It can be seen from Table XI that there was a slight, but constant and uniform increase in the weight of the kidneys of the injected rats in every litter. With the large doses the effect was greater, though not exceeding 20·8 %, than

with the small doses of 0·25 D. and 0·5 D., with which the greatest increase in weight was 9·8 % (per 200 g. of body weight). These changes would therefore seem to be due to the influence of testicular hormone and not accidental.

The influence on adrenals, thyroid, thymus, liver, spleen and heart.

Only the general averages of the changes in weight of these organs are given in Table XII, these changes being, in most cases, small and irregular. Unless the histological examination of these organs, which is now in progress, reveals something special, it will not be possible to draw any positive conclusions.

Table XII. *Influence on adrenals, thyroid, hypophysis, thymus, liver, spleen and heart of normal rats.*

Average percentage changes in weight (actual and per 200 g. of body weight) of organs of the injected rats as compared with those of the control rats.

Organs	Batch of extract	Actual weights			Weights per 200 g. of body weight		
		0·25 D.	0·5 D.	2 D.	0·25 D.	0·5 D.	2 D.
Adrenals	I	+11·4	+ 5·1	+ 5·1	+13·6	+ 1·5	+ 8·5
	II	—	+11·6	− 3·2	—	+ 5·8	− 0·4
Thyroid	I	+ 2·0	+ 3·0	+17·2	+ 4·5	+ 0·5	+20·7
	II	—	+12·1	− 3·0	—	+ 6·1	+ 0·5
Hypophysis	I	+ 4·1	− 0·6	− 5·0	+ 6·1	− 4·3	− 2·5
	II	—	+ 8·7	+29·2	—	+ 2·4	+33·3
Thymus	I	+ 5·1	+ 6·3	−25·6	+ 7·9	+ 4·7	−22·8
	II	—	+23·9	−32·1	—	+16·8	−30·4
Liver	I	+ 0·3	− 1·1	− 6·2	+ 3·3	− 3·5	− 2·8
	II	—	+ 5·8	− 5·8	—	− 0·1	− 3·3
Spleen	I	+14·1	+ 5·9	− 7·0	+17·0	+ 3·3	− 4·5
	II	—	+ 3·1	+13·2	—	− 2·2	+16·2
Heart	I	− 1·0	+ 4·1	− 1·6	+ 1·7	+ 2·0	+ 2·2
	II	—	+ 6·7	+ 1·2	—	+ 0·8	+ 4·7

Some of the changes observed, however, should be noted. Large doses of both extracts increased the speed of involution of the thymus in all the 6 rats injected with this dose, the average for litter 8 being by 22·8 % and for litter 12 by 30·4 % (Table XII).

The percentage changes in the weights of the other organs are included in Table XII in order to show how small are the physiological variations of most of the average weights of the organs of different groups of normal rats, which, in the present and previous papers, has already been shown to be true for castrated rats. The accuracy of the method, therefore, would seem to be also satisfactory for normal rats. The results obtained would probably have been still more satisfactory had we not been handicapped in their interpretation by the use of two batches of the extracts which had different properties and strengths. This is shown in Table XII; *e.g.* the injection of 2 D. of batch II increased the weight of the hypophysis on the average by 33·3 % and of 2 D. of batch I the weight of the thyroid by 20·7 %. These changes, although each the effect of one of the batches only, were nevertheless found in all the rats injected with this batch. Thus, the weights (per 200 g. of body weight) of the hypophysis, in the 3 control rats of litter 12 were 7·0, 6·9 and 6·9 mg. and in the 3 injected rats 8·5, 10·1 and 9·0 mg.; and the weights of the thyroid of the 2 control rats of litter 8 were 19·1 and 21·4 mg. and of the injected rats 23·3, 25·7 and 24·5 mg. The occurrence of such similar changes in

all the rats of a group can hardly be accidental. The absence of the corresponding changes from the two other litters injected with the same dose but from a different batch is probably due to differences in the properties of the two batches due to a different degree of purification.

Discussion.

In interpreting the results obtained an important reservation must be made. The experiments were performed with two preparations isolated from human urine. One of these preparations was less purified but had a stronger action on the sexual organs. The other preparation seemed to be highly purified but was still not a chemically pure substance. In both preparations, therefore, impurities were present, which substances may have an action on the same organs and functions as those influenced by the testicular hormone.

The specificity of the result obtained.

(*a*) *On castrated rats.* The recognition of the action of the testicular hormone and that of the impurities is comparatively easy in castrated rats, since all the changes produced in rats by castration due to the absence of the internal secretion of the sexual glands are sufficiently well known. It is probable that all the changes towards a normal condition, produced in the organs of the castrated rat, are due to the testicular hormone, whilst unexpected effects should be considered to be at least doubtfully due to this.

(*b*) *On normal rats.* On the other hand we have no definite knowledge of pure uncomplicated cases of hypersecretion of testicular hormone in human patients, nor have we yet a chemically pure testicular hormone for use in experiments on normal animals.

The following data may, however, be used in the elucidation of this problem. The sexual hormone of the anterior lobe of the hypophysis has no action on castrated animals. Its activity is, therefore, assumed to be due to its stimulating action on the testes, or to activation of the hormone secreted by the testes. If this is true the changes produced in normal male rats by the injection of anterior lobe hormone must be due to the direct influence of the testicular hormone.

In young rats when small or moderate doses of anterior lobe hormone are injected the size and weight of the testes are unchanged or slightly increased, while, histologically, maturation of the seminiferous tissue and proliferation of Leydig's cells are found [Kraus, 1930; Boeters, 1931; Moore and Price, 1931]. With larger doses given for a long period, the seminiferous tissue is damaged, but proliferation of the Leydig's cells is still produced. The testes are found to have increased in weight (explained by proliferation of the Leydig's cells [Boeters, 1931] or to have decreased owing to degenerative processes in the seminiferous tissue [Kraus, 1930]. The testes of adult rats show no changes and are resistant to the influence of the hormone of the anterior lobe.

In both young and adult rats the size and weight of the prostate with seminal vesicles are increased by the injections.

However, Borst *et al.* [1931], Gostimirovic [1932], Neumann and Peter [1931] found that only the combination of two hormones of the anterior lobe, isolated from urine, Prolan A (follicle stimulating) and Prolan B (luteinising) were able to bring about an increase in the size of the prostate with seminal vesicles. Prolan A alone, when injected for a long period, depressed

the development of the prostate and seminal vesicles, these organs being found to be smaller than in the uninjected control animals.

Korenchevsky [1930, 1], by extracting bull's hypophysis with glycerol, prepared extracts which, in normal rats, also decreased the weight of the testes by about 12 % and of the prostate with seminal vesicles by about 23 %. The weight of the penis was not influenced by the injections. The effect of this extract, when injected into cryptorchid rats, was much more pronounced, the weights of the testes, prostate with seminal vesicles and even the penis decreasing by about 31 %, 62 % and 31 % respectively.

The weights of the thyroids decreased in both normal and cryptorchid rats after injection of the glycerol extract, but no changes were found in castrated rats [Korenchevsky, 1930, 1]; whilst the injections of extracts prepared in the usual way produced hypertrophy and hyperactivity of the thyroids in guinea-pigs [Aron, 1929; Loeb and Basset, 1930] and in dogs [Houssay, Biasotti and Mazzocco, 1932], but not in rats [Loeb and Basset, 1930; Aron, 1930].

As far as we know the effects of glycerol extracts of the hypophysis have not been studied by other workers, but Korenchevsky, from the results quoted above, suggested that there is present in the hypophysis a depressing principle, antagonistic to the male gonad-stimulating hormone. Biedl [1927] also prepared an extract of hypophysis which had a similar depressing influence on the testes and prostate with seminal vesicles.

Moore and Price [1932] injected an extract of testicular hormone into normal young and adult rats. The testes of the young rats were found to be decreased in weight by about 48 % to 69 %, while the germinal epithelium was damaged. Moore concludes also that the prostate and seminal vesicles in his experiments were stimulated by the testicular hormone, the average increase of the seminal vesicles being from 12 % to over 700 %. His results, however, do not always support this conclusion; *e.g.* in the series of experiments on p. 37, of the 8 injected animals in 3 the seminal vesicles were decreased and in 5 increased in size. The 3 rats in which the seminal vesicles were decreased were injected with only 2, 3 and 5 CU respectively. The results given by Moore and Price rather suggest that the very large increase in weight of the prostate with seminal vesicles was produced by the injection of large doses of testicular hormone (12 CU or more).

Moore also injected 10 adult normal rats with testicular hormone. Unfortunately the figures are not given, but it follows from the description that changes were absent or very small. Moore explains this failure of the adult rat to respond to testicular hormone by insufficiency of the amount injected, although 6 to 21 CU were injected daily for 20 days.

Moore suggests that the gonad hormones of either sex exert a depressing effect on the hypophysis, whilst the hypophyseal hormone stimulates the sexual glands and that without this hormone development of testes is impaired. We offer the alternative suggestion of the direct influence on the gonads of the depressing principle, which seems to be present in the anterior lobe, the occurrence of which has been demonstrated by Biedl and by Korenchevsky and by the experiments with Prolan A.

The results obtained with young normal rats.

When all the facts mentioned above, and their possible explanations, are taken into consideration, the results we obtained on young normal rats are seen to be consistent. Thus, in agreement with Moore and Price, we obtained a decrease in the weight of the testes. These decreases of about 33 % to 58 %,

obtained by us with large doses similar to those used by Moore and Price, do not differ much from the figures of 48 % to 69 % given by Moore. A stimulation and increase in the weight of the prostate with seminal vesicles was also obtained with large doses.

Small doses, in our experiments, resulted in less interference with the development of the testes, while the prostate with seminal vesicles and penis were found, in most cases, to be slightly decreased. Although similar changes were not taken into account by Moore, they occurred in some of his rats. Therefore, although these might be within the limits of physiological fluctuations, they seem to be significant, especially since our present results are in agreement with those showing the influence of anterior lobe extracts. Thus, after injections of these latter extracts a decrease was noted in the size of the testes and according to the size of the dose an increase or decrease in the size of prostate with seminal vesicles.

The different effects of the small and large doses might be explained tentatively by the domination of the effect of the depressing principle of hypophyseal hormones with small doses and of the stimulating hormone with large doses. However, in the absence of more numerous special experiments and a chemically pure hormone it is not possible to give a definite explanation of the facts observed.

The changes of the other organs were not investigated by other workers. Those obtained by us in the weight of penis, kidneys, thymus and fat deposition were small and their probable significance has been discussed in previous paragraphs. The small changes in weight of the remaining organs which we investigated were inconsistent.

Summarising the results obtained on young normal rats, testicular hormone injected for 3 weeks in the doses used in the experiments depressed the development of the testes and apparently with some doses even of the secondary sexual organs; *i.e.* was able to produce some pathological changes in young animals.

The results obtained by us with castrated rats.

Changes, which may be defined as specific for testicular hormone, *i.e.* the return of the pathological condition of castrated rats towards normal, were obtained in the prostate with seminal vesicles (the weights of which increased by a maximum of 900 %); in the penis (increase in weight reaching 172 %); in the thymus (considerable return to the normal speed of involution, the decrease in weight reaching a maximum of 47 %); in the adrenals (decrease in weight up to 43 %) and, with small doses only, in the thyroid (increase in weight up to 21 %).

Besides these changes improvement in appetite, increase in the weight of the liver (maximum 29·1 %) and of the kidneys (maximum 30·9 %) must be considered specific for testicular hormone since these changes were shown in every group of injected rats. Unfortunately, up to the present we have omitted to study the influence of castration on the liver and kidneys, nor were we able to find any data in the literature referring to this subject.

There was, however, in our experiments no return towards normal of two significant changes found in castrated rats, the increased fat deposition and the hypertrophy of the hypophysis.

The changes in the body weight in relation to appetite, the rebuilding towards normal of the sexual organs and of the thyroid and the increase in weight of the liver and kidneys all indicate the anabolic properties of the testicular hormone. Thus we did not find either with young castrated rats

(previous experiments) or with adult castrated rats, any changes which would indicate that the injections produced any effect that was not beneficial.

This does not seem to be true in the case of young normal animals which have a natural, abundant internal secretion of the sexual glands.

The method of assay.

In these, as in previous, experiments, small variations from the average weights were found to occur in all the organs investigated, both in castrated and in normal rats, and in injected and in uninjected rats.

The relation of direct proportion between the increase of the dose of the hormone and the average increase in the weight of the prostate with seminal vesicles was also apparent in the present experiments of 3 weeks' duration, the approximation of the experimental and theoretically expected figures being remarkably close.

When this relation was first noticed in the preliminary experiments the striking closeness of the figures was thought to be "probably fortuitous" [Korenchevsky, Dennison and Schalit, 1932, p. 1311]. The fact that in our previous work on the rat unit [1932] and again in the present experiments this definite relation was obtained seems, therefore, to indicate the accuracy of the method of assay and the suitability of the rat unit, based on the changes in weight of the prostate with seminal vesicles as proposed by us in the previous paper.

It is possible that the effect may be proportional to the number of days of injection. Unfortunately the testicular extract used in the previous experiments of 7 days' duration was of a different batch from those used in the present experiments of 21 days, being, however, similarly purified and, according to Schering, of the same strength as batch II. In addition the weights of the rats were different in the two groups of experiments. However, as can be seen from Table V, the percentage increase in prostate with seminal vesicles with 1 D. and 2 D. of batch II, injected for 21 days, was approximately three times that obtained in the previous experiments of 7 days' duration with similar doses (2·5 and 5·0 CU). Thus the average increase in weight of prostate with seminal vesicles per 200 g. of body weight per capon unit per week was in the previous experiments of 7 days' duration 37·5 % and in the present experiments of 3 weeks' duration (Table V) 36·5 % $\left(\frac{274}{2{\cdot}5\times 3}\right)$ and 37·0 % $\left(\frac{555}{5{\cdot}0\times 3}\right)$.

With regard to the penis, the approximate direct proportion between the dose injected and the change obtained was found to be no longer valid when the injections were prolonged from 1 to 3 weeks, the increase in the penis being much less than that expected. The following explanation could be given of this fact. The penis consists of muscular, vascular and connective tissue, the reaction of which to growth stimulus is slower than that of the glandular tissue of the prostate with seminal vesicles, especially since these glands are able to excrete varying amounts of secretion into the glandular lumena, in response to the varying strength of the stimulus. Another important difference is that in castrated rats the degree of atrophy of the penis is much less than that of the prostate with seminal vesicles, the weight of the former being 3 to 4 times, of the latter at least 20 times, less than that in normal rats.

The checking of the rate of growth which would be theoretically expected with the approach to the normal size of the organ would, therefore, be reached much sooner in the case of the penis than in the case of the prostate with

seminal vesicles. The penis, therefore, of castrated animals is similar in this respect to the comb in capons, not only in its structure but also in its response to the injections of testicular hormone. Thus Schoeller and Gehrke [1931] found that with injections of testicular hormone a constant maximum speed of comb-growth in capons was reached with a certain dose and period after which the speed decreased.

The results obtained on the penis suggest that in our experiments with regard to the response of prostate with seminal vesicles, only a small part of the curve relating the dose and the effect was obtained. Probably the increase of the dose or of the period of injections beyond those used in our experiments would change the curve considerably. At present the changes in other organs cannot be accurately related to the dose. This point is discussed in the previous paragraphs on these organs.

The most important feature of the method of assay described is that a comparison is made not of an average of the changes in the individual rats, but of the mean of the average changes in the litters. Could this principle be used in the various other methods proposed for assay it is possible that a closer agreement in the results might be obtained.

Animals for assay.

As in the previous work, in the present experiments these similar results were obtained on a standardised stock of rats prepared for assay in the same way. The comparison of two different stocks of normal untreated animals gave considerable differences in the body weight and, in the few litters investigated, in the weight of prostates with seminal vesicles. It would seem, therefore, desirable that for international biological standardisation a definition of standardised stocks should be worked out, as well as the conditions of preparation of the animals in order that results as nearly comparable as possible should be obtained.

Summary.

1. The changes produced by 21 days of injections of two testicular preparations, extracted from human urine, were studied on 33 young normal male rats belonging to 7 litters and on 29 castrated rats belonging to 5 litters.

2. *Young normal rats.* (*a*) The physiological variations from the average of the organs investigated were small.

(*b*) The injected animals, as compared with the controls, showed depression of the development of the testes in most cases with small doses and in all rats with large doses. The prostate with seminal vesicles and penis were, in most cases, slightly decreased in weight with small doses, whilst with large doses an increase in weight was obtained and also an acceleration in the involution of the thymus and a slight increase in the growth of the kidney. No large or constant changes were found in the weights of the other organs or in the body weight. The appetite of the injected animals was nearly the same or sometimes less than that of the controls.

(*c*) The changes obtained were compared with those produced by the hormones of the anterior lobe of the hypophysis, and the possible relation of these changes to the internal secretion of the anterior lobe was discussed.

3. *Castrated rats.* (*a*) In the injected animals, as compared with the controls, the following changes of a return of the organs towards a normal condition were observed: a large increase in the weight of the prostate with seminal vesicles

and of the penis; a slight increase in the weight of the thyroid with small doses; an increased speed of involution of the thymus and a decrease in the hypertrophy of the adrenals. The injections increased the weight of the liver and of the kidneys, stimulated appetite and produced a larger gain in body weight.

(*b*) As in the previous experiments, which were of short duration, a correlation of direct proportion was apparent between the increase of the dose, from 2·5 to 5·0 CU, and the degree of average increase in weight of the prostate with seminal vesicles.

(*c*) It is possible that the effect on prostate with seminal vesicles may also be proportional to the number of days of injections.

(*d*) In the case of the penis, this direct relation between dose and increase in weight, which was found to be true in a less sensitive degree in the short duration experiments, was no longer evident after the injections made for a longer period.

(*e*) A probable explanation of this difference in the responses of the prostate with seminal vesicles and of the penis was suggested.

(*f*) The anabolic properties of testicular hormone on castrated rats were discussed.

4. The sufficiency of the degree of accuracy of the method of assay was corroborated, and the importance was emphasised of comparing the mean of the average changes in the litters and not of the average changes in the individual rats.

5. Sesame oil solution of testicular hormone was found to be suitable for assay on rats.

6. The differences in body weight and the weights of prostate with seminal vesicles of the rats belonging to two different stocks were demonstrated.

7. For international biological assay the desirability of using standardised stocks of animals bred and kept under the same conditions and similarly prepared for assay was suggested.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work and to them our thanks are due.

REFERENCES.

Aron (1929). *Compt. Rend. Soc. Biol.* **102**, 682.
—— (1930). *Compt. Rend. Soc. Biol.* **105**, 581.
Biedl (1927). *Arch. Gyn.* **132**, 175.
Blyth, Dodds and Gallimore (1931). *J. Physiol.* **73**, 136.
Boeters (1931). *Virchow's Arch.* **280**, 215.
Borst, Doederlein and Gostimirovic (1931). *Münch. med. Woch.* **78**, 19.
Champy (1931). *Compt. Rend. Soc. Biol.* **108**, 367.
Gostimirovic (1932). *Sitz. Ber. ges. Morph. Phys., München*, **40**, 83.
Houssay, Biasotti and Magdalena (1932). *Compt. Rend. Soc. Biol.* **110**, 834.
—— —— and Mazzocco (1932). *Compt. Rend. Soc. Biol.* **110**, 832.
Kabak (1931). *Endokrinologie*, **9**, 84.
Korenchevsky (1922). *Med. Res. Counc. Spec. Rep. Ser.* No. 71, p. 8.
—— (1930, 1). *Biochem. J.* **24**, 383.
—— (1930, 2). *J. Path. Bact.* **33**, 607.
—— (1932, 1). *Biochem. J.* **26**, 413.
—— (1932, 2). *Biochem. J.* **26**, 1300.

Korenchevsky, Dennison and Kohn-Speyer (1932). *Biochem. J.* **26**, 2097.
—— —— and Schalit (1932). *Biochem. J.* **26**, 1306.
—— Schalit and Graetz (1932). *Biochem. J.* **26**, 423.
Kraus (1930). *Proc. II. Internat. Congress for Sex Research*, p. 451.
Loeb and Basset (1930). *Proc. Soc. Exp. Biol. Med.* **27**, 490.
Moore and Price (1931). *Amer. J. Phys.* **99**, 197.
—— —— (1932). *Amer. J. Anat.* **50**, 13.
Neumann and Peter (1931). *Zentr. Gynaek.* **55**, 2670.
Sampson and Korenchevsky (1932). *Biochem. J.* **26**, 1322.
Schoeller and Gehrke (1931). *Wien. Arch inn. Med.* **21**, 329.
Smith and Chick (1926). *Biochem. J.* **20**, 131.

LXXVII. SPECIFICITY OF HEXURONIC (ASCORBIC) ACID AS ANTI-SCORBUTIC FACTOR[1].

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(*Received March 6th, 1933.*)

Although it has been shown that specimens of hexuronic acid[2] possess anti-scorbutic properties [Svirbely and Szent-Györgyi, 1932, 1; Harris, Mills and Innes, 1932; Zilva, 1932, 2] it has remained to determine whether this activity is an inherent property of the pure substance itself or ascribable to some associated impurity. So often in the past has the vitamin activity of an apparently pure and crystalline material been shown later to be due either to some major admixture (*e.g.* in the case of the original preparation of calciferol) or else to traces of a contaminant (as with vitamin B_1) that the issue was by no means a foregone conclusion. Indeed, from the start Zilva [1932, 1] has opposed the idea that hexuronic acid could be identical with the vitamin, and more recently [1932, 3] has contended that there is in fact no consistent relationship between antiscorbutic activity and reducing capacity—one of the most distinctive properties of hexuronic acid.

The steps leading up to the present position may be summarised as follows. Szent-Györgyi [1928] demonstrated the existence in various vegetable and animal tissues of a naturally occurring reducing substance; he succeeded in isolating it, and named it hexuronic acid (from its supposed analogy with the glycuronic acids). As he pointed out in his first paper on the subject [1928] hexuronic acid appeared to be identical with a reducing substance known to occur in active vitamin C concentrates. Unfortunately however the matter was pursued no further at the time because observations by Zilva seemed to show that the vitamin was independent of the reducing principle. At about the same time, Tillmans, in the course of his work as Public Analyst for Frankfurt, to distinguish between fresh and stale or natural and artificial foods measured their reducing capacity, employing for the purpose the well known oxidation-reduction indicator 2:6-dichlorophenolindophenol. He reported that natural, but not "artificial," lemon juice reduced the indicator [Tillmans, Hirsch and Reinshagen, 1928]; and in further series of papers Tillmans and his collaborators [1932] drew attention to the fact that there existed a considerable degree of parallelism (with some irregularities) between the antiscorbutic activity of foods or vitamin C preparations and their reducing capacity. The conclusion was definitely drawn that "a reducing substance is the 'carrier' of vitamin C activity" [Tillmans, Hirsch and Jackisch, 1932] and in a paper published in March 1932 attention was specifically drawn to Szent-Györgyi's hexuronic acid as having similar pro-

[1] Communicated to the Biochemical Society, February 17th, 1933 [Birch, Harris and Ray, 1933].

[2] Since renamed ascorbic acid.

perties to the reducing substance in question, and being "possibly identical" with it [Tillmans, Hirsch and Dick, 1932]. Shortly after this, in April 1932, a preliminary note by Svirbely and Szent-Györgyi [1932, 1] mentioned that a test was in progress to discover whether hexuronic acid possessed antiscorbutic properties, and a little later [1932, 2] it was recorded that hexuronic acid, when fed at the level of 1 mg. per day had protected guinea-pigs over the usual 90 day test period. (Other levels of dosage were not tried.) Simultaneously with the contribution of Svirbely and Szent-Györgyi, King and Waugh [1932], of the University of Pittsburgh, announced that they had succeeded in isolating vitamin C and that it turned out to have the properties of hexuronic acid. It may perhaps be mentioned here that Szent-Györgyi's colleague Svirbely had but recently been in collaboration with King in some of the later stages of his work on the concentration of the vitamin.

From their evidence that a dose of 1 mg. had protected guinea-pigs for 90 days, Svirbely and Szent-Györgyi [1932, 2] concluded that "vitamin C is a single substance and identical with hexuronic acid." This conclusion was immediately combated by Zilva [1932, 1] who stated that he had himself obtained fractions active in doses of 0·5 mg. (*cf.* 1 mg. for hexuronic acid) which were "evidently grossly contaminated"—a fact which "militated against the contention that hexuronic acid and the antiscorbutic factor are identical." Actually the limited evidence available seemed insufficient to warrant a final decision in either direction.

In favour of the theory that hexuronic acid was identical with the vitamin, King and Waugh [1932] could point to the fact that the final stages of their isolation process led to a product of constant chemical composition. Svirbely and Szent-Györgyi [1932, 1] advanced the argument that the amount of hexuronic acid presumed to be present in orange juice was of the right order to account for its activity. Since, however, the amount of hexuronic acid in orange juice was not known with any certainty, no method for its estimation being in fact yet available (the amount actually present being as we now know about double the amount imagined), and since also the activity of hexuronic acid itself had not yet been determined, the necessity for further work was fully evident. Earlier papers from this laboratory [Harris, Mills and Innes, 1932; Harris and Ray, 1932; 1933; Birch, Harris and Ray, 1933] have given a preliminary account of experiments which definitely supported the theory of the identity of hexuronic acid and vitamin C. The detailed results, together with further supporting evidence, are set out in the present paper.

Experimental.

Our experimental evidence is best presented as a series of separate lines of testimony, as follows. (1) The antiscorbutic activities of several natural sources were in the first place shown to run parallel with their apparent hexuronic acid contents as roughly indicated by the yields of the latter recoverable from them. (2) Specimens of hexuronic acid derived from different sources were next shown to have identical antiscorbutic activities. (3) This activity remained unchanged after various repurification processes. (4) The hexuronic acid contents of a large variety of foodstuffs, as determined by a chemical titration method were shown to account exactly in all cases for their observed activities. (5) Hexuronic acid as measured by the intensity of the absorption band at 265 $m\mu$ likewise accounted for observed activity. (6) In guinea-pigs deprived of vitamin C, the hexuronic acid disappeared from suprarenals or liver coincidently with loss of vitamin C

activity. (7) In dogs or rats on the other hand, examples of species which are able to thrive in the absence of vitamin C, antiscorbutic activity and hexuronic acid remained unimpaired together. (8) The rate of destruction of hexuronic acid by oxidation, heat and alkali was similar to that of antiscorbutic activity. (9) Hexuronic acid was synthesised concurrently with vitamin C activity in the plant.

1. *Correlation between antiscorbutic activity and hexuronic acid recoverable.*

In the first paper on this question from this laboratory [Harris, Mills and Innes, 1932] it was pointed out that on the basis of the hexuronic acid theory the suprarenal gland, although not previously recognised as having any antiscorbutic activity, should be intensely active, since this organ was a better source of hexuronic acid even than such active materials as orange or lemon juice or cabbage. This was found to be the case, and full results have already been published [Harris and Ray, 1932] which showed that ox suprarenal cortex had three times the activity of orange juice, in exact accord with the fact that its apparent hexuronic acid content, as judged by the yield recoverable, was likewise three times as great. As we pointed out, there is a considerable loss of hexuronic acid during the recovery process, but about the same fraction is lost in the two cases. Our conclusions have been confirmed by further investigations, both upon the hexuronic acid content determined chemically and upon the antiscorbutic activity as measured by more than one method. The following figures may be cited in illustration[1].

Hexuronic acid content of ox suprarenal [Birch, Harris and Ray, 1933]	1·85 mg. per g.
Hence calculated "minimum protective dose" (*i.e.* amount containing 0·9 mg.)	0·49 g.
"Minimum protective dose" found (*i.e.* biologically equivalent to 1·5 cc. orange juice)	0·5 g.

2. *Constant activity of hexuronic acid from different sources.*

A number of specimens of hexuronic acid, derived from both an animal and a vegetable source (*viz.* suprarenal and paprika) have been examined for antiscorbutic activity under the same conditions. As is shown by Fig. 1 (typical curative tests), no difference could be detected. Coincident results were obtained also in a series of experiments in which the activity was determined by the alternative tooth-structure technique and by preventive tests. Table I gives results by the former method for hexuronic acid from paprika. A similar preliminary test on hexuronic acid from ox suprarenals has already appeared [Harris, Mills and Innes, 1932], and as shown by Fig. 2, the ratio between the effective doses of orange juice and the two materials is the same: 1 g. of hexuronic acid from suprarenal was equivalent to 1·5 cc. of orange juice in giving almost complete protection in an 11 day test, and 2 g. of hexuronic acid from paprika to 3·0 cc. of orange juice in a 15 day test.

Degree of activity of hexuronic acid. This question has already been opened elsewhere [Harris and Ray, 1932]. Our preliminary results showed uniformly that the amount of hexuronic acid equivalent to 1 cc. of orange juice was greater than 0·33 and less than (or equivalent to) 0·66 mg., the best value being 0·6 mg. Confirmatory experiments (see, *e.g.*, Fig. 2) give values from 0·6 to 0·66 mg. The "minimum protective dose" of hexuronic acid, equivalent to 1·5 cc. of orange

[1] Additional work on the vitamin activity and hexuronic acid content of suprarenals from various species will be published in a later paper.

juice hence works out at 0·9 (or 1·0) mg. This agrees exactly with the amount of hexuronic acid present in orange juice, as determined chemically (see below). At this point we would wish to suggest the adoption of a specimen of hexuronic

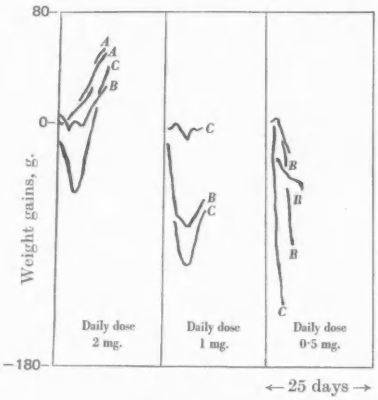

Fig. 1.

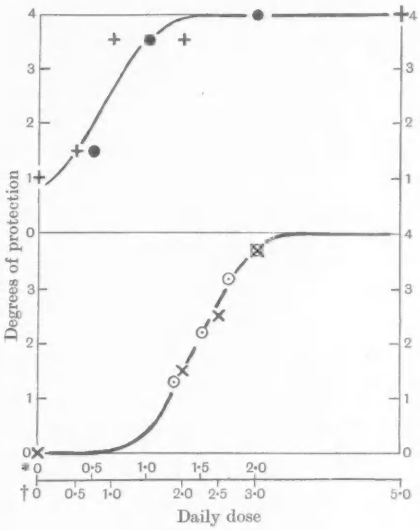

Fig. 2.

Fig. 1. Curative tests on guinea-pigs, showing equal activity of hexuronic acid derived from different sources, or repurified and recrystallised. *A* = Hexuronic acid from suprarenal cortex, crude; *B* = Hexuronic acid from paprika; *C* = Hexuronic acid from paprika, repurified and recrystallised.

Fig. 2. Equivalent activity of hexuronic acid from suprarenal (above) and paprika (below). Comparisons with orange juice as standard of reference. ● Hexuronic acid from suprarenals, + Orange juice, 11 day test; ⊙ Hexuronic acid from paprika, × Orange juice, 15 day test.

* Scale for hexuronic acid, mg.; † Scale for orange juice, cc.

Table I. *Comparison of antiscorbutic activities of hexuronic acid (from paprika) and orange juice by the tooth-structure method.*

Amount of material fed per day	Degree of protection
Hexuronic acid, 2 mg.	4, 3, 4 (av. 3·7)
,, 1·75 mg.	2–3, 3, 4 (av. 3·2)
,, 1·5 mg.	2, 2–3 (av. 2·2)
,, 1·25 mg.	1, 2, 1 (av. 1·3)
Orange juice, 3·0 cc.	4, 3, 4 (av. 3·7)
,, 2·5 cc.	2–3, 2–3, 2–3 (av. 2·5)
,, 2·0 cc.	1–2, 1, 2 (av. 1·5)
,, 0 cc.	0, 0 (av. 0)

acid as international standard of vitamin C activity. The advantage of substituting a definite uniform preparation, which can be preserved dry in a stable condition at constant activity, in place of the present standard (any specimen of lemon juice), would seem obvious.

3. *Constant activity of repurified hexuronic acid.*

We have had in our hands a number of specimens of hexuronic acid, of varying degrees of purity, iodine titrations of which have shown, *e.g.* values from 93 to over 99 %. Our results showed that a given amount of hexuronic acid always had the same activity irrespective of the presence of impurities. We were fortunate enough to be able to examine also a specimen of hexuronic acid which, after having been repurified by Prof. Szent-Györgyi, was further recrystallised at the Birmingham laboratory, and kindly put at our disposal by Dr E. L. Hirst, who described it as being in an extremely high state of purity. Again the activity showed no difference from that of the original specimens (see Fig. 1). This was confirmed in a second set of assays.

4. *Antiscorbutic activities of foods determined from their hexuronic acid contents.*

In an earlier paper [Harris and Ray, 1932] a method was described for estimating hexuronic acid chemically, and it was shown that the hexuronic acid content of orange juice exactly accounted for its antiscorbutic potency. The same result was obtained with several other materials including lemon juice, grape-fruit juice, tomato juice and pineapple juice. The method has since been adapted for use on a micro-scale by our colleague Mr T. W. Birch [Birch, Harris and Ray, 1933] and a large variety of foodstuffs has since been tested, covering the widest possible range of biological activities, with relative values varying from about 4 to 300. From these analyses we have calculated the antiscorbutic activities of the various materials, again on the basis of the relation that 0·6 mg. of hexuronic acid is equal in antiscorbutic potency to 1 cc. of

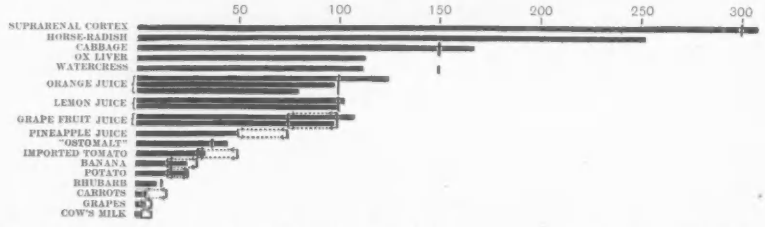

Fig. 3. *Antiscorbutic activities of foods.* Lengths of columns represent activities as calculated from hexuronic acid content, estimated chemically. Cross lines represent values of activities as determined biologically.

orange juice. It is perhaps the most compelling evidence that could be offered for the hexuronic acid theory, that in all cases the vitamin C value thus calculated from the hexuronic acid content agreed with the value as determined biologically. This is shown graphically in Fig. 3. The variation in antiscorbutic potency of different varieties of apples even is quantitatively confirmed from the hexuronic acid content (Table II).

5. *Agreement between ultra-violet absorption at 265* mμ *and antiscorbutic activity.*

The presence of an ultra-violet absorption band at 265 $m\mu$ [Bowden and Snow, 1932] suggested another means of checking the correlation between hexuronic acid and vitamin C activity. Observations by our colleague Dr W. J.

Table II. *Antiscorbutic value of apples.*

	Calculated from hexuronic acid content	Reputed value (determined biologically)
Bramley's Seedling, peel	128	150
Newton Wonder, peel	40	50
Bramley's Seedling, cortex	27	50–100
Newton Wonder, cortex	8	15
Edward VII, cortex	3	<7

Dann showed that in many natural sources it was unfortunately impossible to measure intensity of absorption at 265 $m\mu$ direct owing to the masking of the band by absorption in the same region by other constituents. It is possible however to make use of this criterion in the case of hexuronic acid or vitamin C concentrates, and also in certain classes of food materials; and measurements of absorption intensities upon lemon juice, orange juice, grape-fruit juice, and hexuronic acid itself, by Dr Dann gave values in good accord with our titration results and biological determinations (Table III).

Table III. *Hexuronic acid content.*

	As determined from intensity of absorption at 265 $m\mu$ (W. J. Dann)	As estimated by titration	As calculated from anti-scorbutic activity
Lemon juice	0·86	0·62	0·6
Orange juice	0·66	0·6–0·75	0·6
Grape-fruit juice	0·40	0·6	0·45–0·6

6. *Coincident disappearance of hexuronic acid and vitamin C activity during scurvy in guinea-pigs.*

In earlier papers [Harris and Ray, 1932; 1933] we reported that suprarenals of normal guinea-pigs had intense antiscorbutic activity, and that this activity disappeared with the development of scurvy. This loss of antiscorbutic activity ran parallel with the disappearance of hexuronic acid. This was demonstrated both qualitatively, by means of Szent-Györgyi's silver staining method (carried out by Dr Thomas Moore) [Moore and Ray, 1932] and quantitatively by analyses by the titration method (carried out by Mr T. W. Birch) (see Table IV). A

Table IV. *Effect of vitamin C intake on the hexuronic acid content of suprarenals in guinea-pigs.*

Amount of vitamin C received per day as supplement to scurvy-producing diet (in mg. of hexuronic acid)	Amount of hexuronic acid remaining in suprarenal, after 14 days on diet (in mg. per 1 g. of gland)
0	0·21
1	0·43
2	0·62

similar loss of hexuronic acid occurred from the liver, which again ran parallel with its loss of antiscorbutic activity in scurvy. Our biological estimations showing the loss of antiscorbutic activity have already been published [Harris and Ray, 1933].

7. *Synthesis of hexuronic acid parallel with vitamin C in other species.*

One of us [Harris, 1932] has recently shown that dogs are able to synthesise their own vitamin C when fed for prolonged periods on vitamin C-free diets. This has been confirmed in a further series of tests. Liver from such dogs administered to scorbutic guinea-pigs was found to cure the latter of scurvy (Fig. 4), in pronounced contrast with the effect of liver from guinea-pigs which

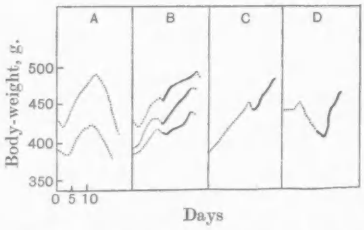

Fig. 4. Guinea-pig feeding tests, demonstrating synthesis of vitamin C by the dog. A, Negative controls; B, Extract of liver from dogs on vitamin C-free diet; C, Extract of liver from dog on complete diet; D, Positive control (5 g. cabbage per diem). ..... Vitamin C-free diet; —— Experimental diet.

had been similarly fed on a vitamin C-free diet. The suprarenals from these dogs were also tested in the same way, and, although not enough material was available for a long test, definite evidence of antiscorbutic activity was again obtained. Similar feeding tests with the suprarenals of rats kept on a vitamin C-free diet will be discussed in a later paper[1]. Chemical tests, both silver staining and estimation by the titration method on the suprarenals or livers of these two species, showed that the maintenance of vitamin C activity was associated with the presence of hexuronic acid, a result which should be compared with the simultaneous loss of activity and hexuronic acid in the guinea-pig.

8. *Destruction of hexuronic acid by oxidation, heat and alkali.*

The data given in Table V show that the conditions which favour the destruction of hexuronic acid are the same as those which are known to determine the destruction of vitamin C activity. That is, destruction is favoured by aeration, by alkalinity and by heat. The actual rate of destruction of hexuronic acid under different conditions of p_H, oxygen access, and temperature agree with what data are available for vitamin C activity. Where oxygen was rigidly excluded hexuronic acid was surprisingly stable to heat even in presence of alkali, which again agrees with the behaviour of the vitamin. Oxidation and not heat or alkalinity *per se* was the determining factor bringing about destruction, although of course such oxidation was accelerated with increased p_H and rise of temperature. There was evidence that when hexuronic acid was boiled in alkaline solution the amount destroyed depended on such "accidental" variables as: (1) the amount of oxygen already dissolved in the solution and (2) the oxygen which gained access to the solution after the boiling had stopped and before the titration could be begun. Under these circumstances it has been found difficult

[1] The suprarenal of the rat appears to be very rich in vitamin C, compared *e.g.* with that of the normal guinea-pig, a fact which may be of significance in view of the circumstance that the rat but not the guinea-pig is able to synthesise the vitamin.

Table V. *Effect of heat, aeration and p_H on destruction of hexuronic acid.*

	Percentage of hexuronic acid undestroyed (as determined by titration)
Orange juice, fresh	100
" boiled 2 hours	34
" aerated 2 hours	79
" unheated	100
" heated on water-bath, 1 hour	60
" " 2 hours	30
Solution of hexuronic acid, at p_H 3·4	100
" heated ½ hour	82·8
" heated 1 hour	
Solution of hexuronic acid, at p_H 4·5	100
" heated 1 hour	16·5
" heated 2 hours	0
Solution of hexuronic acid, before treatment	100
" after 15 minutes' aeration in alkali	9
" after 30 "	0

to make direct quantitative comparisons between the exact rates of destruction of hexuronic acid and antiscorbutic potency. There can however be no doubt that there is good agreement both qualitatively, in the sense that the conditions which destroy hexuronic acid are the same as those which inactivate the vitamin, and also quantitatively, in the sense that the rates of destruction can be shown to be at least approximately parallel.

9. *Coincident synthesis of hexuronic acid and antiscorbutic activity by the plant.*

It is well known that in the vegetable kingdom synthesis of vitamin C occurs during germination. We have been able to show that hexuronic acid appears concurrently, as measured by this titration method (Table VI is representative of several experiments).

Table VI. *Synthesis of hexuronic acid during germination.*

	Hexuronic acid content	
	mg. per g.	mg. in single seed or seedling
Seed peas, before germination	0·00	0·00
" soaked for 24 hrs. (not germinated)	0·08	0·02
" " 48 hrs. (germinated)	0·69	0·21
" " 72 hrs. (germinated)	0·82	0·26
" " 96 hrs. (germinated)	0·86	0·27
Oatmeal, ground	0·00	—
Oats, whole grain	0·11	—
" after 96 hrs. germination	0·20	—
" after 120 hrs. germination	0·42	—

Discussion.

The results given in the experimental section may be expressed by the generalisation that a given amount of hexuronic acid invariably possesses the same biological activity. This statement applies, as we have shown, not only to

its distribution throughout nature (over 30 natural sources, with relative hexuronic acid contents ranging from 1 to 300 having been examined), but also to the specimens of hexuronic acid recovered from various sources (and representing only a fraction of the full amount present), to preparations of hexuronic acid repurified through chemical means and then repeatedly recrystallised, to its disappearance and synthesis in the animal and plant organisms, and to its destruction by chemical means. Now, we found full antiscorbutic activity for specimens of hexuronic acid which were at least 99 % pure, judging by our titration curve results [Birch and Harris, 1933], by iodine titration, or by elementary analysis. In other words, therefore, one alternative only remains to our conclusion that hexuronic acid is identical with the vitamin. That is, it would be necessary to assume that wherever hexuronic acid is found or however it is treated it invariably occurs combined with the same unvarying but minute amount (less than 1 %) of some unspecified contaminant—*i.e.* that this hypothetical substance accompanies it throughout nature, and, in unalterable proportion, clings to it throughout the process of recovery, and is synthesised with it, is utilised with it and is destroyed with it. In the absence of any positive evidence in favour of such a supposition (and it would appear unreasonable to interpret so extended an association as a mere matter of coincidence) it seems only logical to conclude that hexuronic acid is itself the vitamin.

Summary.

Experiments have been carried out with the object of ascertaining whether antiscorbutic activity is an inherent property of hexuronic acid itself or due to some associated impurity. The evidence is consistently in favour of the former view, an unvarying and very extensive correlation having been established between hexuronic acid and antiscorbutic activity, as follows:

1. In the first place it was shown that the antiscorbutic activities of several natural sources were approximately proportional to the amounts of hexuronic acid recoverable from them: thus suprarenal cortex (ox), not hitherto recognised as an antiscorbutic, had three times the potency of orange juice.
2. Specimens of hexuronic acid from different sources (suprarenal; paprika) were assayed biologically and found to have identical antiscorbutic activity.
3. Hexuronic acid after repurification processes and recrystallisation always maintained its original activity.
4. The hexuronic acid contents of over 30 different natural sources (with relative hexuronic acid contents varying from 1 to 300) as determined chemically (see following paper) were shown to account quantitatively for their known antiscorbutic activities.
5. In the case of several materials it was also possible to estimate the hexuronic acid content by intensity of absorption at 265 $m\mu$ (carried out by W. J. Dann), and this result was also in agreement with the biological and chemical tests.
6. The rate of destruction of hexuronic acid under varying conditions of aeration, heat and alkali was found to resemble that of antiscorbutic activity.
7. In the guinea-pig the antiscorbutic activity of the suprarenal (or liver) was lost with the development of scurvy and this coincided with the disappearance of hexuronic acid.
8. With other species, the rat or dog, which are able to synthesise their own vitamin when none is provided in the diet, the antiscorbutic activity and the hexuronic acid both remain unaffected.

9. Hexuronic acid is synthesised concurrently with antiscorbutic activity by the plant on germination.

The antiscorbutic activity of hexuronic acid has been determined by several alternative methods (curative, tooth-structure and preventive). The amount equivalent to 1·0 cc. of orange juice is greater than 0·33 mg. and less than (or equal to) 0·66 mg.; best value = 0·6 mg.; or "minimum protective dose" = 0·9 mg. per guinea-pig per day. We recommend its adoption as international standard.

Generous gifts of hexuronic acid from Prof. Szent-Györgyi's laboratory have greatly facilitated this work.

REFERENCES.

Birch and Harris (1933). *Biochem. J.* **27**, 595.
—— —— and Ray (1933). *Chem. Ind.* **52**, 159; *Nature*, **131**, 273.
Bowden and Snow (1932). *Nature*, **129**, 720.
Harris (1932). Quoted by Innes, *Ann. Rep. Inst. Animal Path., Cambridge* (1931), 150.
—— Mills and Innes (1932). *Lancet*, ii, 235.
—— and Ray (1932). *Biochem. J.* **26**, 2067.
—— —— (1933). *Biochem. J.* **27**, 303.
King and Waugh (1932). *Science*, **75**, 357.
Moore and Ray (1932). *Nature*, **130**, 997.
Svirbely and Szent-Györgyi (1932, 1). *Nature*, **129**, 576.
—— —— (1932, 2). *Nature*, **129**, 690.
Szent-Györgyi (1928). *Biochem. J.* **22**, 1387.
Tillmans and Hirsch (1932). *Biochem. Z.* **250**, 312.
—— —— and Dick (1932). *Z. Unters. Lebensm.* **63**, 267.
—— —— and Hirsch (1932). *Z. Unters. Lebensm.* **63**, 1.
—— —— and Jackisch (1932). *Z. Unters. Lebensm.* **63**, 276.
—— —— and Reinshagen (1928). *Z. Unters. Lebensm.* **56**, 272.
—— —— and Siebert (1932). *Z. Unters. Lebensm.* **63**, 21.
Zilva (1932, 1). *Nature*, **129**, 690.
—— (1932, 2). *Nature*, **129**, 943.
—— (1932, 3). *Biochem. J.* **26**, 1624.

LXXVIII. A MICRO-CHEMICAL METHOD FOR DETERMINING THE HEXURONIC ACID (VITAMIN C) CONTENT OF FOODSTUFFS, *ETC.*[1]

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(*Received March 6th, 1933.*)

In an earlier paper from this laboratory [Harris and Ray, 1933] reference was made to a method of estimating hexuronic acid in foodstuffs, depending on modifications in the use of Tillmans's reduction test. It was shown that the hexuronic acid content of orange juice so determined accounted quantitatively for its antiscorbutic activity, and similar results were alluded to for lemon juice, tomato juice, grape-fruit juice and pineapple juice. As was mentioned, the method has since been adapted to a micro-chemical scale, and the purpose of the present paper is to give the detailed description of the method and results for a large number of common foodstuffs.

Experimental.

Method in outline. A small amount of the foodstuff is weighed out and ground up with sand and sufficient 20 % trichloroacetic acid solution to give a final concentration of the latter of about 5 %. The extract is then made up to a suitable volume: *e.g.* when working with materials of the potency of orange, lemon, *etc.* a dilution to about 1 in 10 is suitable; with less potent materials the dilution should be correspondingly less. The filtered extract is transferred to a micro-burette graduated in 0·01 cc. A measured volume, say 0·05 cc. of a recently prepared standardised solution of 2 : 6-dichlorophenolindophenol (about 0·01 *M*), is placed at the bottom of a small pointed tube. (It may be conveniently run out of Krogh and Keys's [1931] micro-syringe, using the screw gauge, the error with a volume of this order being less than 2 %.) The trichloroacetic acid extract is then run in from the burette until the red colour, which the indicator immediately assumes in acid solution, is just discharged. (The titration should be concluded within about 2–3 minutes, otherwise an error may be caused by the fact that the indicator may sometimes tend to fade slowly in the absence of hexuronic acid.) The amount of extract so needed is noted and, since the indicator is standardised in terms of hexuronic acid itself (see below), the hexuronic acid content of the unknown can be immediately calculated. From the known antiscorbutic activity of hexuronic acid, the antiscorbutic activity of the unknown can then be expressed in terms of guinea-pig dosage

[1] Communicated to the Biochemical Society, February 17, 1933 [Birch, Harris and Ray, 1933].

or compared with the potency of a standard material, as previously described [Harris and Ray, 1933].

Standardisation of indicator. For standardisation, a measured volume of the same indicator solution as was used for the estimation is titrated, likewise in acid solution and under the same general conditions, against a solution of hexuronic acid. The amount of pure hexuronic acid in the solution should in turn be checked (in case the specimen is at all impure) by iodine titration. From this relation, 1 cc. of indicator solution is evaluated as equivalent to so many mg. of hexuronic acid. Practical examples illustrating the use of this standardisation method have already been published [Harris and Ray, 1933]. Hexuronic acid is now available commercially, so that other workers should experience no difficulty in carrying out this necessary standardisation. It would however be legitimate to use fresh lemon juice or orange juice instead, if a more rough comparison only is desired.

Preparation of indicator. The indicator solution is made up by dissolving approximately 0·1 g. of the dye in a small amount of boiling distilled water. The residue left undissolved is filtered off and again extracted with boiling water; the combined extracts are then made up to 50 cc. At times trouble was experienced in getting a satisfactory end-point to the titration, the colour change being from red to brown instead of to colourless or pale yellow. This usually occurred when the indicator solution was several weeks old and was overcome when a fresh solution was made up according to the above procedure.

Extraction process. It may be noted that it was found impossible in most cases to remove anything like the full amount of vitamin by a simple expression process. Much of it seems to be retained within the cell structures. Furthermore, an important advantage of adding the trichloroacetic acid is that it appears to stabilise the vitamin and protect it against often fairly rapid destruction by agencies present in the tissue juices, presumably of an oxidative nature and due largely to enzyme systems. As has already been reported from this laboratory, disintegration of cell structure by freezing similarly promotes a considerable destruction of antiscorbutic activity [Mills, 1932].

Choice of titration reagent and conditions of test. Owing to the fact that hexuronic acid possesses outstanding reducing activity, a reduction test seemed the most hopeful method to try. As has already been reported [Harris and Ray, 1933], iodine proved far too unspecific, even at a strongly acid reaction (a condition which tended to inhibit the activity of certain other reducing agents and leave that of hexuronic acid unimpaired). An oxidation-reduction indicator with constants of such an order as to be unaffected by other naturally occurring reductants "weaker" than hexuronic acid was clearly indicated. Study of the literature showed that several oxidation-reduction indicators had in fact been described which from theoretical considerations could be expected to prove superior even to 2 : 6-dichlorophenolindophenol in this respect. However enquiries from many chemical manufacturers here and abroad showed that these were not to be obtained commercially. We decided accordingly that there was some benefit, from the immediate practical point of view, in using 2 : 6-dichlorophenolindophenol, and in actual experience it has been found to give reliable results.

This indicator has previously been employed by Tillmans and co-workers in their important work on the reducing capacity of foodstuffs and food-extracts. A number of points of difference in our use of it should therefore be noted here. Firstly, our object is to determine quantitatively the actual weight of hexuronic acid present in a given amount of material and not merely to obtain arbitrary

numerical values for relative reduction potentials. Secondly, the simple extraction with trichloroacetic acid seems undoubtedly preferable to the elaborate process of heating with sulphuric acid, evaporation, *etc.*, as used by Tillmans. Even more important is the difference that our titration is to be carried out in acid solution. We have shown that certain naturally occurring reducing agents, including notably glutathione and certain phenolic compounds, tend to reduce the indicator in neutral or alkaline solution [Harris and Ray, 1933]. Hence when working with the many natural sources known to contain, *e.g.* glutathione, it seems essential to work in acid solution if the possibility of deceptively high results is to be ruled out. A number of irregularities are in fact apparent in the proportionality between antiscorbutic activity and Tillmans's reduction values, which do not occur in the case of our measurements of hexuronic acid.

We have examined a large number of reducing agents, and found only one occurring naturally, cysteine, which will appreciably reduce the indicator under the conditions described. If working therefore with stale or autolysed tissues, which may contain measurable amounts of free cysteine, it is necessary to make a control test for the latter, *e.g.* by the Sullivan reaction. Adrenaline also may interfere but it is not likely in practice to be often present in sufficiently large concentration to be of any consequence. Yeast also was found to contain a substance which reduced the indicator. Whether or not it is identical with hexuronic acid we have still to determine.

The procedure for titrating a given amount of reagent with the unknown, instead of the more familiar reverse practice, may perhaps be criticised. However we found that if indicator were run into the unknown in the usual manner there was some difficulty in determining the end-point with precision. This is overcome by the method described, which also gives better results than the use of external indicator.

We hope to publish at a later date an account of measurements by the electrometric method. The relative advantages of alternative indicators are also being investigated, as to the sharpness of the end-point, clearness of the colour change, *etc.*

Degree of accuracy attainable. It was found that the end-point colour change was sufficiently sharp to enable duplicate determinations to agree to 97 % when working with solutions of hexuronic acid itself or natural sources of an activity such as lemon juice, orange juice, grape-fruit or the like. As we have shown, the results, taking materials of the widest range of potency, run parallel with the values determined biologically. The actual sensitivity of the method of course far exceeds that possible by the biological method of assay. With sources containing little vitamin C the percentage error naturally tends to be higher than with more active material, but again it is certainly far less than when determined biologically.

Results.

Table I gives the amount of hexuronic acid, as determined by this method, in various natural sources. From this we have calculated the "theoretical" minimum dose, and compared it with the "known" minimum dose, as determined biologically. The calculated, or theoretical minimum dose, is derived from the hexuronic acid content from the known antiscorbutic activity of hexuronic acid itself. For this purpose 0·9 mg. of hexuronic acid is taken as equivalent to 1·5 cc. of orange juice, or 0·9 mg. of hexuronic acid is taken as the minimum

daily dose to protect guinea-pigs from scurvy, both of which relations are in accordance with the experimental biological results of Harris and Ray [1933; also preceding paper]. In other words we have taken as the "calculated" minimum dose the amount of the material in grams which contains 0·9 mg. of hexuronic acid. Table I shows well the agreement between the known biological values and the values calculated from their hexuronic acid content.

Table I.

	Hexuronic acid content mg. per g.	Minimum daily dose for guinea-pigs, g.	
		As calculated from hexuronic acid chemically determined	As measured biologically (newly determined or reputed value)
Cabbage	1·0	0·9	1
Watercress	0·67	1·3	1
Lemon juice	0·62	1·5	1·5
	0·60	1·5	
Orange juice, several types	0·75	1·2	1·5
	0·59	1·5	
	0·48	1·9	
Grape-fruit juice	0·65	1·4	1·5–2
	0·59	1·5	
Pineapple juice	0·30	3	2–3
Imported tomato, juice of	0·21	4·3	3–5
Banana	0·15	6	5–10
Potato	0·15	6	6–10
Rhubarb	0·059	15	12
Carrots	0·028	32	10–35
Grapes	$\leqq$0·030	$\geqq$30	>20, 40
Imported peach, juice of	0·015	60	—
Horse-radish	1·6	0·6	—
Apples:			
Bramley's Seedling, cortex	0·16	5·5	3–5
" peel	0·77	1·2	1
Newton Wonder, cortex	0·053	17	10
" peel	0·24	3·7	3
Blenheim Orange, cortex	0·031	29	—
" peel	0·33	2·7	—
Edward VII, cortex	0·017	53	>20
" peel	0·12	7·5	2?
Cox's Orange Pippin, cortex	0·016	56	>20
" " peel	0·09	10	—
Suprarenal cortex, ox	1·85	0·5	0·5
Liver, ox	0·68	1·3	—
Milk, cow's (variable)	0·025–0·019	36–47	20–60
"Ostomalt"	0·27	3·3	4
Egg-yolk	0·00	∞	∞
Sussex ground oats	0·00	∞	∞

Summary.

Table I gives the hexuronic acid contents of about 30 food materials and the corresponding "calculated" minimum antiscorbutic doses, which agree well with the minimum doses as actually determined biologically.

The method described entails preliminary grinding with sand and trichloroacetic acid followed by titration of the acid extract against a measured volume of 2 : 6-dichlorophenolindophenol, which has been standardised against a solution of hexuronic acid of known strength, in turn standardised against iodine.

The determination takes only a few minutes to complete; in the case of a material of the activity, *e.g.*, of orange juice, the amount used in titration need be no more than about 0·03 cc., and the sensitivity is such that duplicates agree to within 3 %.

Certain naturally occurring reducing agents, including glutathione, which react with the indicator when the reducing capacity of foods is determined in more nearly neutral solution according to the Tillmans technique do not interfere. Although no claim is made to absolute specificity, it is clear that in practice the method gives accurate results on a large variety of animal and vegetable materials. Many reducing agents were tested with negative results. In the presence of free cysteine, however, as in certain autolysed tissues, a special control is necessary.

REFERENCES.

Birch, Harris and Ray (1933). *Chem. Ind.* **52**, 159.
Harris and Ray (1932). *Biochem. J.* **26**, 2067.
—— —— (1933). *Biochem. J.* **27**, 303.
Krogh and Keys (1931). *J. Chem. Soc.* 2436.
Mills (1932). *Biochem. J.* **26**, 704.

LXXIX. THE TITRATION CURVE AND DISSOCIATION CONSTANTS OF VITAMIN C[1].

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(*Received March 6th, 1933.*)

It is now well recognised that the dissociation constants of an organic compound are characteristic of, and to a considerable extent descriptive of, the different types of groups, or combinations of groups, which it contains. A method of measuring dissociation constants of weak acids, or bases or ampholytes, depending on the determination of the titration curve and differentiation

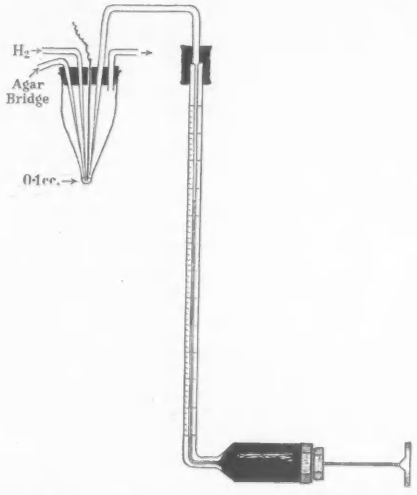

Fig. 1. Micro-apparatus used for electrometric titration of vitamin C.

between the combined and free titrant at each p_H value, was introduced by Harris [1923, 1, 2; 1925]. This method provides in addition a simple means of determining molecular or combining weight. These were the two main con-

[1] Communicated to the Biochemical Society, February 17, 1933 [Birch, Harris and Ray, 1933].

siderations prompting the present work. A third was to obtain information as to the degree of ionisation of hexuronic acid at different p_H values, in order to discover whether this could be correlated with observations by our colleague W. J. Dann on the changes in ultra-violet absorption at varying reactions.

Experimental.

The principle of the method was that described in earlier papers [Harris, 1923, 1, 2; 1925; Birch and Harris, 1930]. The work was carried out on a micro-scale using only 0·10 cc. of solution to be titrated (containing 10–12 mg. of hexuronic acid). The apparatus, which has previously been used for determining the titration curve of crystalline vitamin B_1 preparations (in amounts of about 2 mg.) is shown in Fig. 1. The micro-burette used, based on that described by Rehberg [1925], is actuated by the displacement of the titrant from a capillary by means of a mercury thread. Measurements were taken against a saturated KCl calomel half cell, standardisation being effected by measuring the E.M.F. difference with the H-electrode in 0·1 *N* HCl solution, assumed to have a p_H value of 1·07. The blank correction at each p_H value was calculated from the relation

$$[\text{Free NaOH}] = \gamma\,[OH^-] = \gamma\,\frac{Kw}{[H^+]},$$

γ depending on the total sodium concentration and being taken as 0·7 [*cf.* Harned, 1925].

Table I. *Titration of 12·00 mg. of hexuronic acid (94 % pure by iodine value) with 0·105 N NaOH (temp. 16–18°).*

NaOH found for equivalence point = 0·613 cc.
NaOH required by theory = 0·610 cc.

(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)	(*g*)	(*h*)
	cc. of 0·105 *N* NaOH			NaOH in equivalents			
p_H	Added	Blank correction	Corrected for blank	Uncorrected	Corrected for blank	$p_{K_{a_1}}$	$p_{K_{a_2}}$
2·08	0·0000	—	—	0·000	—	—	—
2·69	0·0020	—	—	0·003	—	—	—
3·03	0·0052	—	—	0·084	—	—	—
3·40	0·0102	—	—	0·167	—	4·10	—
3·62	0·0152	—	—	0·248	—	4·10	—
3·85	0·0210	—	—	0·343	—	4·13	—
4·13	0·0300	—	—	0·490	—	4·14	—
4·33	0·0375	—	—	0·613	—	4·13	—
4·61	0·0450	—	—	0·735	—	4·17	—
4·91	0·0519	—	—	0·848	—	4·16	—
5·48	0·0583	—	—	0·952	—	—	—
5·98	0·0600	—	—	0·981	—	—	—
8·11	0·0613	—	—	1·000	—	—	—
9·39	0·0620	—	—	1·013	—	—	—
9·74	0·0630	—	—	1·030	—	—	—
10·12	0·0650	—	—	1·062	—	—	—
10·43	0·0680	—	—	1·111	—	—	—
10·88	0·0750	—	—	1·225	—	—	11·42
11·11	0·0811	—	—	1·325	—	—	11·43
11·28	0·0870	—	—	1·422	—	—	11·42
11·51	0·0948	—	—	1·549	—	—	11·44
11·65	0·1000	—	—	1·633	—	—	—
11·94	0·1102	0·002	0·108	1·800	1·76	—	—
11·27	0·1200	0·004	0·116	1·961	1·89	—	—
12·48	0·1263	0·007	0·119	2·063	1·94	—	—
12·70	0·1350	0·012	0·122	2·205	2·01	—	—
12·88	0·1450	0·019	0·126	2·368	2·06	—	—

Two determinations were carried out on two different specimens of hexuronic acid, the first 94 % pure and the second over 99 % pure.

Table II. *Titration of 10·315 mg. of pure recrystallised hexuronic acid (iodine value 103 %) with 0·105 N NaOH (temp. 16·5–18·0°).*

NaOH found, for equivalence point = 0·0554 cc.
NaOH required by theory = 0·0558 cc.

(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)	(*g*)	(*h*)
	cc. of 0·105 *N* NaOH			NaOH in equivalents			
p_H	Added	Blank correction	Corrected for blank	Uncorrected	Corrected for blank	pK_{a_1}	pK_{a_2}
2·12	0·0000	—	—	0·000	—	—	—
2·54	0·0015	—	—	0·027	—	—	—
2·83	0·0028	—	—	0·051	—	—	—
3·10	0·0050	—	—	0·090	—	—	—
3·36	0·0081	—	—	0·146	—	—	—
3·51	0·0117	—	—	0·211	—	—	—
3·70	0·0149	—	—	0·269	—	4·14	—
3·85	0·0191	—	—	0·345	—	4·13	—
3·99	0·0230	—	—	0·415	—	4·14	—
4·13	0·0269	—	—	0·486	—	4·15	—
4·30	0·0313	—	—	0·564	—	4·19	—
4·39	0·0346	—	—	0·624	—	4·17	—
4·53	0·0384	—	—	0·693	—	4·18	—
4·82	0·0452	—	—	0·816	—	4·18	—
4·96	0·0474	—	—	0·856	—	4·19	—
5·08	0·0490	—	—	0·885	—	4·20	—
5·19	0·0502	—	—	0·906	—	4·21	—
5·36	0·0517	—	—	0·933	—	4·21	—
5·59	0·0530	—	—	0·957	—	—	—
5·83	0·0540	—	—	0·975	—	—	—
6·09	0·0545	—	—	0·984	—	—	—
6·51	0·0549	—	—	0·991	—	—	—
7·30	0·0553	—	—	0·998	—	—	—
8·97	0·0558	—	—	1·007	—	—	—
9·78	0·0570	—	—	1·027	—	—	—
10·20	0·0584	—	—	1·053	—	—	—
10·46	0·0600	—	—	1·083	—	—	—
10·89	0·0655	—	—	1·182	—	—	11·54
11·20	0·0722	—	—	1·303	—	—	11·56
11·48	0·0801	—	—	1·446	—	—	11·57
11·60	0·0840	—	—	1·516	—	—	11·57
11·72	0·0882	0·0010	0·0872	1·592	1·574	—	11·59
11·84	0·0921	0·0013	0·0908	1·663	1·639	—	11·59
11·96	0·0959	0·0018	0·0941	1·731	1·699	—	11·59
12·11	0·1000	0·0026	0·0976	1·805	1·762	—	11·60
12·23	0·1040	0·0035	0·1005	1·877	1·814	—	11·59
12·36	0·1080	0·0049	0·1031	1·949	1·861	—	11·57
12·52	0·1120	0·0072	0·1048	2·022	1·892	—	11·60
12·68	0·1170	0·0105	0·1065	2·112	1·923	—	11·60
12·80	0·1212	0·0139	0·1073	2·188	1·937	—	—
12·94	0·1280	0·0202	0·1078	2·311	1·946	—	—
13·07	0·1352	0·0280	0·1072	2·441	1·935	—	—

Results.

The combining weight as determined agreed with the theoretical, for $C_6H_8O_6$, with an error of less than 1 %. The p_H readings fall with great precision on the theoretical curves with the constants shown (Figs. 2 and 3). The two specimens

gave constants in good agreement. (We attach little importance to the suggestion in Fig. 2 of a third group titrating (*i.e.* for the less pure specimen). Any small error in absolute measurement of p_H at this highly alkaline reaction

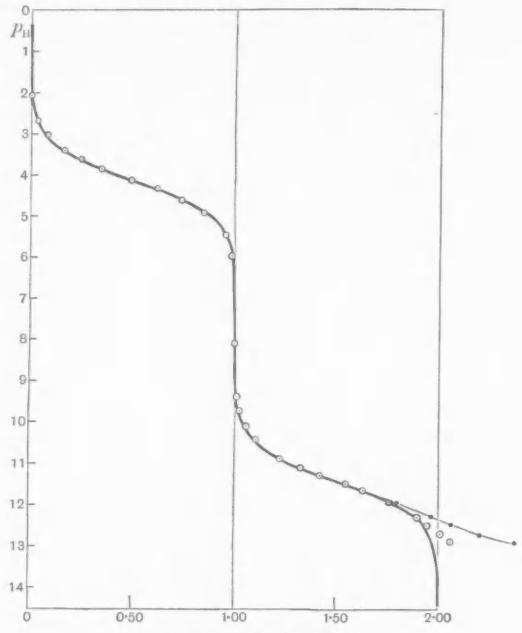

Fig. 2. Titration curve of hexuronic acid (first specimen).

——— Theoretical for $p_{K_{a_1}} = 4{\cdot}14$
$p_{K_{a_2}} = 11{\cdot}43$

⊙ Experimental, corrected • Experimental, uncorrected

Table III.

	First determination (specimen 1)	Second determination (specimen 2)
$p_{K_{a_1}}$	4·14 (0·05 *M*, $t = 17{\cdot}5°$)	4·17 (0·04 *M*, $t = 17{\cdot}5°$)
$p_{K_{a_2}}$	11·43 (0·04 *M*, $t = 18°$)	11·57 (0·03 *M*, $t = 18°$)

would appreciably affect the error of the blank correction and so the accuracy of the extreme end of the corrected curve. Further, the curve for the pure specimen (Fig. 3) shows a very clear end-point to the titration of the second group.)

$p_{K_{a_1}}$ is of the order expected for an α-substituted carboxylic acid. $p_{K_{a_2}}$ may be compared with 10·7 for the keto-enol group in acetoacetic ester, and fructose 11·7 (*cf.* glucose 12, sucrose 13, glycerol, glycol, *etc.* 14).

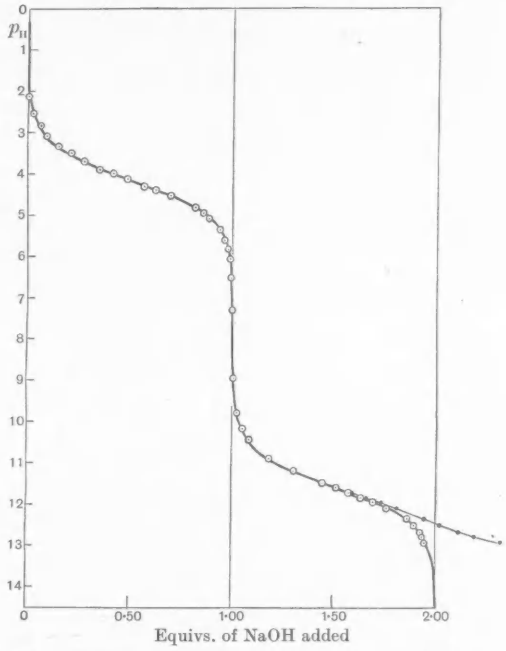

Fig. 3. Titration curve of hexuronic acid (second specimen).

——— Theoretical for $p_{K_{a_1}} = 4{\cdot}17$, $p_{K_{a_2}} = 11{\cdot}57$

⊙ Experimental, corrected • Experimental, uncorrected

Summary.

The titration curve of hexuronic acid has been determined, by a micro-method, using 0·1 cc. of solution. The dissociation constants were

$$p_{K_{a_1}} = 4{\cdot}17; \qquad p_{K_{a_2}} = 11{\cdot}57; \qquad p_{K_{a_3}} \text{ (if any)} = > 14.$$

The combining weight agreed with the formula $C_6H_8O_6$ with an error of less than 1 %.

REFERENCES.

Birch and Harris (1930). *Biochem. J.* **24**, 564.
—— —— and Ray (1933). *Chem. Ind.* **52**, 159.
Harned (1925). *J. Amer. Chem. Soc.* **47**, 676.
Harris (1923, 1). *Proc. Roy. Soc. Lond.* B **95**, 440.
—— (1923, 2). *J. Chem. Soc.* **123**, 3294.
—— (1925). *Nature*, **115**, 119.
Rehberg (1925). *Biochem. J.* **19**, 270.

LXXX. INVESTIGATIONS ON THE NATURE OF HAEMOPOIETIN, THE ANTI-ANAEMIC SUBSTANCE IN HOG'S STOMACH. I.

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(*Received February 15th, 1933.*)

Introduction.

The successful treatment of pernicious anaemia depends upon the use of a diet containing fresh liver or liver extracts, or fresh or desiccated hog's stomach [Minot and Murphy, 1926; Wilkinson, 1930].

The nature of the active anti-anaemic principle in liver has been investigated by many workers. West and Howe [1930] and Dakin, West and Howe [1930] reported the isolation from liver extract of an active amorphous substance which they regarded as a dipeptide of *l*-γ-hydroxyproline and β-hydroxyglutamic acid; this claim was later, however, withdrawn [West and Howe, 1931]. Cohn, Minot and their co-workers [1927; 1928; 1929; 1930, 1, 2] obtained an active amorphous solid from liver extract by removing the proteins with either alcohol or basic lead acetate and precipitating with phosphotungstic acid when it was found that the material obtained from the acetone-soluble phosphotungstates was rich in the active principle. Further concentration was achieved by fractionating with various mixtures of alcohol, ether and water, when finally an intensely active amorphous substance was obtained of which the injection of only 0·025 g. daily was sufficient to give a maximum reticulocyte response in a case of pernicious anaemia. This substance has been shown to be neither a protein, lipin, carbohydrate, α-amino-acid nor polypeptide, and Cohn *et al.* [1930, 2] are of the opinion that it is probably an ω-amino- or imino-acid.

The use of preparations of hog's stomach is a direct consequence of the finding of Castle [1929] and his co-workers [1930; 1931; 1932] that normal human gastric juice, after incubation *in vitro* with the proteins of beef muscle, was able to induce remissions in cases of pernicious anaemia. Neither the gastric juice nor the proteins of beef muscle administered separately, however, were active. It was suggested, therefore, that there was an "intrinsic factor" of enzymic nature in normal gastric juice which, reacting with the proteins of beef muscle (the "extrinsic factor"), gave rise to haemopoietically active material. Wilkinson [1930] has, moreover, shown that when normal human gastric juice is given with protein food to patients suffering from pernicious anaemia, a reticulocyte response does occur although the juice is quite inactive when administered alone.

These experiments led one of us [Wilkinson, 1930] to consider the use of stomach tissue itself as a source of the active anti-anaemic principle. It was

found that responses could be obtained with hog's stomach but not with the stomachs of the ox or sheep.

The superiority of the hog's stomach treatment over the older liver therapy is manifested not only by the quickness with which normal health is reached but also by the greater speed of remission of the disease as measured by the increase in the number of red blood cells and in the percentage of haemoglobin [*cf.* Wilkinson, 1930; 1931, 1, 3]. This suggests either that the stomach is a richer source of the active anti-anaemic principle than liver, or else that the active principles in these two organs are not identical, though there must evidently be some fairly close relationship between them. Our experiments on the nature of the active anti-anaemic principle in hog's stomach support the second of these possibilities.

Several factors were found to destroy the stomach active principle and consequently led to the formation of inactive stomach extracts. Among the most striking of these were (*a*) exposure to temperatures greater than 45°, (*b*) autolysis, (*c*) prolonged digestion with pepsin or trypsin. Moreover, it was not found possible to obtain active stomach extracts by methods of extraction analogous to those employed in the case of liver.

It appeared, therefore, that the active principle of stomach, for which we have temporarily suggested the name "haemopoietin" [Wilkinson and Klein, 1932], was much more unstable and had different properties from the active principle in liver. Later experiments showed that an active press juice could be prepared from hog's stomach by the method used by Buchner [1897; 1898] to obtain zymase from yeast, and, by precipitating the juice with alcohol, a protein fraction was obtained in which the whole haemopoietic activity of the juice appeared to be concentrated. The active principle of liver, on the other hand, is always found in the filtrate obtained after the removal of proteins [*cf.* Cohn *et al.*, 1928]. This protein fraction from the stomach press juice is highly active peptically but, by dissolution in $N/10$ hydrochloric acid followed by fractional isoelectric precipitation, we have isolated a product which, while haemopoietically active, contains relatively little pepsin. There is, moreover, abundant experimental evidence that the active principle in hog's stomach is certainly not pepsin or rennin [Castle, 1929; Wilkinson, 1930, 1932, 1; Wilkinson and Brockbank, 1930]. The experiments described in this paper indicate that even the relatively mild methods employed for the preparation of concentrates of haemopoietin cause some loss of activity, and in consequence the possibility of isolating it in a chemically pure state seems at present to be remote.

Experimental.

Clinical technique.

The investigation of the potency of the various fractions obtained from hog's stomach involves feeding experiments carried out on well-authenticated and specially controlled cases of pernicious anaemia and the observation of the effect of such treatment in producing firstly a sharp rise or peak in the number of reticulocytes in the circulating blood, followed subsequently by an increase in the number of red blood cells and in the percentage of haemoglobin. The cases used for this purpose were all carefully chosen according to the rigid criteria that have already been laid down by one of us [Wilkinson, 1932, 1].

As an illustration of the use of this method, the results obtained with one of our active fractions, P 5, are shown in Fig. 1. It will be observed that there was a maximum reticulocyte response on the 13th day of treatment, followed by

a gradual fall to normal values with significant rises in both the red cell count and the haemoglobin percentage.

The clinical method, though necessarily crude and possessing the disadvantages of involving the use of relatively large quantities of each fraction and

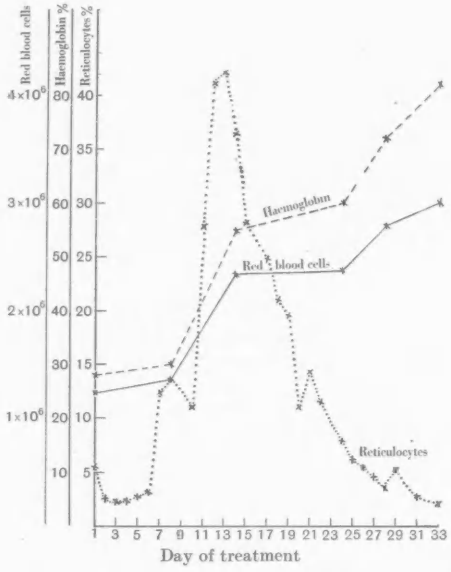


Fig. 1. Effect of fraction P 5 (5 g. per day) on a case of pernicious anaemia.

requiring the use of human test material, is the only one at present available. Although in the course of our work on the treatment of pernicious anaemia with hog's stomach some 400 patients have passed through the hands of one of us (J. F. W.), we have been considerably handicapped by the scarcity of suitable cases for experimental treatment with our various fractions.

Preparation of haemopoietically active desiccated stomach powder.

Active preparations of desiccated hog's stomach are prepared [*cf.* Wilkinson, 1932, 1] by desiccating minced whole hog's stomach, freed from fat by dissection, in thin layers at 40°. The drying must be carried out as quickly as possible and on no account must a temperature of 45° be exceeded.

Desiccated hog's stomach powder is a light, sparingly soluble biscuit-coloured powder, 1 g. of which is equivalent haemopoietically to approximately 4–5 g. of fresh whole hog's stomach. It has strong peptic activity and is active in the treatment of pernicious anaemia, the effective daily dose being 10–30 g. In the course of this work we have frequently had to use much larger amounts of material than we could prepare ourselves and have been kindly supplied with almost unlimited quantities of a very active preparation "Pepsac" made according to our directions by Messrs Boots Pure Drug Co., Ltd., of Nottingham, whom we take this opportunity of thanking.

Peptic activities of preparations from hog's stomach.

Wilkinson [1932, 1] has shown that the pepsin content of desiccated preparations of hog's stomach appears to run parallel with the haemopoietic activity since any conditions which inactivate pepsin during the desiccation also destroy the effectiveness of haemopoietin. Peptic activity determinations have therefore been carried out on all the preparations and fractions described in this paper and the results show that, whilst the above observation holds for preparations of desiccated hog's stomach, peptic activity and haemopoietic activity do not always go hand in hand in the case of other fractions.

Negative attempts to obtain active stomach extracts.

Large scale extractions were tried not only with fresh stomach tissue but also with the more easily handled desiccated stomach powder (p. 602). All the methods of extraction used cannot be described here, but brief details of a few typical experiments are appended by way of illustration.

1. Various methods of extracting both fresh and desiccated stomach were investigated using water, dilute hydrochloric acid at different p_H values, with or without the addition of pepsin or glycerol, but in every case the extracts were inactive whilst the solid or semi-solid residues when separated and desiccated at 40° were still active.

2. The use of organic solvents likewise failed to furnish any active extracts.

Thus, 4500 g. of desiccated hog's stomach were extracted with ether in a Soxhlet apparatus which removed about 10 % of its weight of haemopoietically inactive fat. Subsequent extraction at room temperature with aqueous ethyl alcohol of varying strengths (60–70 %)[1] gave extracts which deposited very small amounts of a wax-like, sparingly soluble lipoid solid (M.P. 175–185°) on cooling in the refrigerator. The extracts and the solid were haemopoietically inactive. The residual stomach powder, however, was still as active as the original powder even after 7 months' continuous extraction with aqueous alcohol. Other organic solvents (*e.g.* glycerol and acetone) similarly failed to yield active extracts.

Preparation of a haemopoietically active press juice from hog's stomach.

In our experiments with stomach tissue a special apparatus was constructed for us by Mr H. W. Baker of the Engineering Department of this University, enabling us to dispense with the use of a cloth bag. Pressure was applied by means of a Tensile and Compressive Testing Machine in the Engineering Department [Wilkinson and Klein, 1932].

The finely minced fresh stomach tissue (1300 g.) was thoroughly mixed with an equal weight of Calais sand and ground in a Bailey's Edge-Runner Mill (about 2–3 minutes) to the correct consistency. The mixture was then packed into the above apparatus with two thicknesses of fine brass gauze and one thickness of stout cloth at the top and bottom, and a pressure of 2–2½ tons per square inch gradually applied until no further quantities of liquid could be obtained. In a series of experiments in which the pressure was 2½ tons per square inch the yield of juice was between 560 and 670 cc. per kg. of fresh tissue. In another series of experiments, using a lower pressure (2 tons per square inch), the yield varied between 525 and 640 cc. per kg.

If the mucosa of hog's stomach was used instead of the whole stomach, a considerably smaller yield of press juice was obtained. In two typical experiments, for example, the yields of juice per kg. of mucous membrane were 305 and 340 cc. (pressure = 2 tons per square inch). This yield was improved somewhat

[1] Alcohol concentrations are given throughout this paper in volume percentages.

by increasing the proportion of sand to mucous membrane from 1 : 1 to 1½ : 1, when the yield varied between 390 and 435 cc. per kg.

Properties of press juice from hog's stomach.

The press juice is a reddish, opalescent, rather viscid liquid containing a small amount of suspended matter which can be removed by filtration through cloth. It decomposes rapidly within 24 hours at ordinary temperatures but can be kept for a few days in a refrigerator.

It has p_H about 6·2. It coagulates with heat owing to the presence of proteins (1·6–2·0 g. of protein per 100 cc. of juice, by Esbach's method). The total solids, determined by heating to 100° for 4 hours, amounted to 6·1 % and the ash content was 0·5 %.

The press juice has a good pepsin content but contains no rennin, amylase, lipase, urease or trypsin.

The following protein tests were positive (*cf.* Table II): biuret, Molisch, lead acetate sulphur test, Millon, Folin phenol, Pauly, Adamkiewicz, *p*-dimethylaminobenzaldehyde, ninhydrin, Sakaguchi and xanthoproteic.

Precipitates were obtained with the following protein precipitants: alcohol, acetone, phosphotungstic acid, picric acid, metaphosphoric acid, trichloroacetic acid, tannic acid, hydroferrocyanic acid, sulphosalicylic acid, lead acetate, mercuric chloride, ferric chloride, colloidal ferric hydroxide, uranium acetate, ammonium sulphate and zinc sulphate.

The press juice was found to be very active in the treatment of pernicious anaemia, using 150 cc. daily, representing about 250 g. of fresh stomach tissue.

Fractionation of press juice with alcohol.

The action of various protein precipitants (alcohol, acetone, tannic acid, picric acid and lead acetate) on press juice has been investigated but so far only the use of alcohol has given satisfactory results.

When the filtered press juice was slowly poured with stirring into 5 volumes of 92 % alcohol, there was a copious flocculent precipitate P 5, which was collected, washed with absolute alcohol and with ether and dried in vacuum desiccators over sulphuric acid. The usual yield of fraction P 5 was 15–18 g. per 500 cc. of press juice, 20 g. being thus derived from approximately 1 kg. of fresh whole stomach tissue.

If the press juice from the mucous membrane of hog's stomach was precipitated in the same way with alcohol, a rather higher yield of fraction P 5 (mucosa) was obtained, namely, about 18–22 g. per 500 cc. of juice.

Properties of fraction P 5. Fraction P 5 is a light cream-coloured powder which gave results on analysis corresponding with those one would expect to obtain from a somewhat impure protein (C, 43·1, 43·3; H, 6·8, 6·7; N, 13·5, 13·5; P, 1·2, 1·2 %. Ash, 5·4, 5·7 %). The ash was found to contain calcium, sodium, potassium, iron and phosphate. Traces of copper, magnesium and sulphate were present but chloride was absent.

Fraction P 5 gave positive results with all the protein reagents mentioned above. It was strongly active peptically, but rennin, amylase, urease, lipase and trypsin were absent. It was only partially soluble in water. Enzyme preparations precipitated by means of alcohol are often sparingly soluble in water [Albert and Buchner, 1900]. Other neutral solvents such as 50 % alcohol, 10 % glycerol and 1 % sodium chloride were less satisfactory than water. It was found, however, that almost complete solution could be achieved by trituration with

cold dilute hydrochloric acid, 1 g. of fraction P 5 requiring about 40 cc. of $N/14$ or 25 cc. of $N/10$ hydrochloric acid. These solutions gave precipitates with all the usual protein precipitants.

Fraction P 5 is very active in the treatment of pernicious anaemia, a dose of only 5 g. daily being adequate as compared with 30 g. daily of desiccated stomach powder. It is evident, therefore, that a considerable concentration of haemopoietin has been achieved in the preparation of this fraction, which is the most active stomach preparation hitherto obtained.

The preparation of fraction P 5 in large quantities was rather laborious, and we were fortunate to secure the co-operation of Messrs Boots Pure Drug Co., who very kindly prepared this fraction for us on a large scale by the method described above.

By precipitating the press juice obtained from the mucous membrane of hog's stomach (p. 603) with alcohol a fraction P 5 (mucosa) was obtained, which was similar in its properties to the ordinary fraction P 5, though it appeared to be slightly more active peptically and haemopoietically.

Alcoholic filtrates from fraction P 5. The combined alcoholic filtrates from one series of experiments (56 litres, representing 9·5 litres of press juice) were evaporated *in vacuo* below 35° to a syrup. This, after keeping for several days in a vacuum desiccator over sulphuric acid, gave 164 g. of a yellowish-brown semi-solid mass, B 5.

Fraction B 5 had a strong porcine odour and was almost completely soluble in water, the solution being acid to litmus. On boiling the solution, no coagulation took place. It had no peptic activity, and of the above-mentioned protein reactions, positive results were given only with the Molisch, Folin phenol, Pauly, ninhydrin and xanthoproteic tests; it failed to respond to the biuret test. The usual protein precipitants likewise gave no precipitates. Phosphotungstic acid produced a white precipitate but this reagent precipitates organic bases and amino-acids as well as proteins. The nitrogen content was low (N, 5·8, 5·9 %). The whole of this nitrogen was of a non-protein character, a high proportion being in the amino-form (found by micro-Van Slyke method: NH_2-N 3·2, 3·2 %). Part of the nitrogen was present as ammonium salts since there was a distinct odour of ammonia on shaking with cold caustic soda. The ash content was 13·6 %; it contained calcium, sodium, potassium, a slight trace of iron, and chloride, sulphate and phosphate.

Fraction B 5 (in doses of 5 g. daily, equivalent to about 500 g. of fresh stomach tissue) was found to be inactive in the treatment of pernicious anaemia. It is evident, therefore, that when the press juice from hog's stomach is treated with alcohol, haemopoietin is entirely precipitated with the protein fraction.

Fractional precipitation of press juice with alcohol.

Fractional precipitation with alcohol was used by Sherman and Schlesinger [1913] for the preparation of amylase from malt extract. The principle of the method is to add alcohol to about 40–50 %, filter off the precipitate and then add further quantities of alcohol to about 80–90 %, when a second precipitate appears.

The press juice and alcohol were both cooled to 0° and the alcohol was slowly added to the juice with constant stirring until a concentration of about 40 % was reached. The temperature was not permitted to rise above 20° during the addition of the alcohol. The precipitate (P 5 α) was collected and dried in a vacuum desiccator over sulphuric acid. Filtration at this stage was extremely

slow, consequently the filtrate had to be kept in the refrigerator overnight and the next stage carried out on the following day. The filtrate was treated with further quantities of alcohol until a concentration of about 80 % was attained when a second precipitate (P 5β) was obtained. This was washed with absolute alcohol and ether and dried in a vacuum desiccator over sulphuric acid.

The average yields of these fractions from 500 cc. of press juice were as follows: P 5α, 7–9 g.; P 5β, 4–5 g.

Properties of fraction P 5α. Fraction P 5α is a yellowish-brown powder giving all the usual protein tests. It is only slightly soluble in water and in N/18 hydrochloric acid. Its nitrogen content is 13·3 %. It has no peptic activity and is not active in the treatment of pernicious anaemia.

Properties of fraction P 5β. Fraction P 5β is a yellowish powder giving all the usual protein tests. It is moderately soluble in water and almost completely soluble in N/18 hydrochloric acid. It contains a higher percentage of nitrogen (13·8 %) than either fraction P 5 or fraction P 5α. It is about as active peptically as fraction P 5 and is also active in the treatment of pernicious anaemia, a case receiving 2–3 g. daily for 13 days showing considerable improvement.

Inactivation of haemopoietin by acetone.

Fraction P 5 is almost completely soluble in N/10 hydrochloric acid, but this acid solution no longer gives a precipitate with alcohol, only a turbidity being produced. Acetone, on the other hand, readily gives a copious white precipitate, P 5A, about 10 g. being obtained from 20 g. of fraction P 5.

Fraction P 5A responds to the usual protein tests, contains 13·9 % N, and has a high pepsin content. It was, however, rather surprising to find that it was unable to induce remissions in cases of pernicious anaemia, the acetone used in the course of the preparation having apparently inactivated the haemopoietin.

Fractionation of fraction P 5 by isoelectric precipitation.

It was hoped to achieve a separation of pepsin and haemopoietin in solutions of fraction P 5 by precipitating out most of the pepsin at its isoelectric point. Various values are given in the literature for the isoelectric point of pepsin, ranging from 2·3 to 3·8 [*cf.* Michaelis and Davidsohn, 1910; Pekelharing and Ringer, 1911; Forbes, 1927; Fenger and Andrew, 1927; Northrop, 1930]. The cause of these variations appears to be the degree of purity of the sample of pepsin used, the presence of proteins and inorganic salts in particular having a marked effect on the value of the isoelectric point. Maximum precipitation of the pepsin in fraction P 5 appears to take place at p_H 4·2, which is somewhat higher than the highest value given above.

In a typical experiment, 20 g. of fraction P 5 were dissolved in 500 cc. of N/10 hydrochloric acid at 20–25°. The p_H of this solution was 2·3. A solution of N NaOH was then run in drop by drop from a burette with constant stirring. The variations in p_H during this addition were as follows:

After adding 10 cc. of N NaOH, p_H = 2·4,
,, 20 ,, ,, p_H = 3·5,
,, 25 ,, ,, p_H = 4·2,
,, 26 ,, ,, p_H = 4·2,
,, 27 ,, ,, p_H = 4·2,
,, 28 ,, ,, p_H = 4·2.

At this stage, the formation of a slimy gelatinous yellowish precipitate, P 5 (i), appeared to have reached a maximum and the mixture was kept

in the refrigerator for half an hour and filtered through a series of No. 41 Whatman papers. Filtration was rather slow. The filtrate from fraction P 5 (i) was brought up to p_H 6·2 by careful addition of *N* NaOH (9 cc.) and then poured with stirring into about 4 volumes of 92 % alcohol cooled to 0°. After standing half an hour in the refrigerator, the precipitate P 5 (ii) was collected.

Fractions P 5 (i) and P 5 (ii) were both washed with absolute alcohol and dried *in vacuo* over sulphuric acid. 20 g. of fraction P 5 usually gave about 5–6 g. of fraction P 5 (i) and about 3–4 g. of fraction P 5 (ii). The yields of these fractions and also the quantities of caustic soda used in the preparation varied somewhat with the particular batch of fraction P 5 that was being used.

An attempt to achieve a separation of fractions P 5 (i) and P 5 (ii) by a simpler and more rapid method than the above, namely, the extraction of fraction P 5 with a buffer solution at p_H 4·2, did not give sufficiently good yields to warrant further investigation. Thus, 15 g. of fraction P 5 were extracted with 400 cc. of a sodium acetate-acetic acid buffer at p_H 4·2. A peptically active residue of 10 g. was obtained but the extract, after precipitation with alcohol, yielded only a very small fraction weighing less than 1 g.

Properties of fraction P 5 (i). Fraction P 5 (i) was a fawn-coloured powder giving all the usual protein reactions. It was only very sparingly soluble in water (N, 13·7 %, soluble N, 1·9 %). It contained 0·75 % P and 3·2 % ash. The ash gave a very strong iron reaction (thiocyanate test) and contained also traces of sodium, potassium, calcium and sulphate. Phosphate was also present. Fraction P 5 (i) was extremely active peptically but contained no haemopoietin.

Properties of fraction P 5 (ii). Fraction P 5 (ii) was a pale buff powder giving all the usual protein tests. In contrast with fraction P 5 (i) it was almost completely soluble in water (total N, 10·6 %, and total soluble N, 8·6 %). It was remarkable in having a relatively high phosphorus content (3·6 %) and a very high ash content (14·8 %). The ash contained calcium, magnesium, sodium, potassium and phosphate. Chloride and sulphate were absent, and there were only extremely minute traces of iron (thiocyanate test) and copper (sodium diethyldithiocarbamate test). The absence of iron and copper from this fraction, and presumably, therefore, from haemopoietin, is interesting in view of the importance attached to the therapeutic use of these metals in the treatment of secondary anaemias.

Fraction P 5 (ii) was only slightly active peptically, as might be expected since most of the pepsin had been removed by isoelectric precipitation. It gave no blue coloration with hydrogen peroxide and benzidine, thereby indicating absence of enzymes of the peroxidase type. The fraction was active in doses of 5 g. daily in cases of pernicious anaemia.

Although pepsin and insoluble impurities have been eliminated in the preparation of fraction P 5 (ii) the haemopoietic activity does not appear to be any greater than that of fraction P 5, probably owing to inactivation of part of the very sensitive haemopoietin.

Chemical reactions and analyses of preparations from hog's stomach.

The chemical analyses and reactions of the various preparations and fractions from hog's stomach described in this paper are shown in Tables I and II, and a diagrammatic scheme indicating the methods by which these fractions were obtained is shown on p. 609.

Table I. *Analyses of preparations from hog's stomach.*

	Total N % (Kjeldahl)	Soluble N %						Phosphorus %	Ash %
		Total	Peptide	Amino	Non-coagulable	Proteose ($ZnSO_4$ ppt.)	Non-protein		
Stomach press juice	1·2	1·2	0·3	0·1	0·1	0·1	0·3	0·15	0·5
Desiccated stomach	11·5	2·2	1·0	0·5	1·1	0·4	1·1	0·8	3·6
Fraction P 5	13·5	2·6	0·9	0·8	1·6	0·9	0·6	1·5	5·7
Fraction B 5	5·9	5·7	0·1	3·2	5·7	0	5·7	1·4	13·6
Fraction P 5 α	13·3	1·8	0·5	0·4	0·9	0·2	0·8	0·9	4·8
Fraction P 5 β	13·8	8·2	5·0	1·8	1·3	0·3	1·1	1·2	11·9
Fraction P 5 (i)	13·7	1·9	1·25	0·35	1·9	0·9	0·7	0·75	3·2
Fraction P 5 (ii)	10·6	8·6	5·15	0·95	8·2	0	0·9	3·6	14·8

Table II. *Properties of preparations from hog's stomach.*

Test	Press juice from hog's stomach	Desiccated hog's stomach	Fraction P 5	Fraction B 5	Fraction P 5 α	Fraction P 5 β	Fraction P 5 (i)	Fraction P 5 (ii)	Fraction P 5 *A*
Biuret	+	+	+	−	+	+	+	+	+
Molisch	+	+	+	+	+	+	+	+	+
Alkaline lead acetate	+	+	+	Very faint	+	+	+	+	+
Folin phenol	+	+	+	+	+	+	+	+	+
Millon	+	+	+	−	+	+	+	+	+
Pauly	+	+	+	+	+	+	+	+	+
Adamkiewicz	+	+	+	Very faint	+	+	+	+	+
p-Dimethylamino-benzaldehyde	+	+	+	−	+	+	+	+	+
Ninhydrin	+	+	+	+	+	+	+	+	+
Metaphosphoric acid	White ppt.	White ppt.	White ppt.	No ppt.	White ppt.	White ppt.	White ppt.	White ppt.	White ppt.
Trichloroacetic acid	,,	,,	,,	,,	,,	,,	,,	,,	,,
Sakaguchi	+	+	+	−	+	+	+	+	+
Xanthoproteic	+	+	+	+	+	+	+	+	+
Murexide	−	−	−	−	−	−	−	−	−
H_2O_2 + benzidine	Blue colour	Blue colour	Blue colour	No colour	Blue colour	Blue colour	Blue colour	No colour	Blue colour
Peptic activity	+	+	+	−	Very slight	+	+	Slight	+
Time taken to digest egg-albumin	28 min.	10–12 min.	7 min.	No digestion	>60 min.	9 min.	4–5 min.	40 min.	9 min.
Effect on cases of pernicious anaemia	Active	Active	Active	Not active	Not active	Active	Not active	Active	Not active

These tables reveal a number of features of interest the most noteworthy being the following.

(*a*) The close parallelism between protein reactions and haemopoietic activity, *i.e.* all the haemopoietically active products gave the protein colour and other tests.

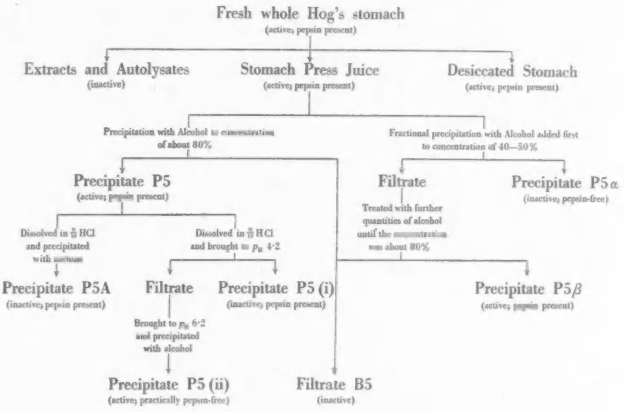
(*b*) Peptic activity and haemopoietic activity are not necessarily related to each other. For example, fractions P 5 A and P 5 (i) were very active peptically but inactive haemopoietically, whereas fraction P 5 (ii) had strong haemopoietic activity but contained very little pepsin.

(*c*) The relatively high proportion of nitrogen (13·5 %) in the haemopoietically active fraction P 5 and the low value for non-protein nitrogen (0·6 %).

(*d*) The low nitrogen content (5·9 %) of the inactive fraction B 5, a very

high proportion of which (3·2 %) was in the amino-form and practically the whole of which was of a non-protein character.

(*e*) The relatively high phosphorus content (3·6 %) of the active fraction P 5 (ii).



Scheme of fractionation.

Summary.

1. Experiments are described on the preparation of extracts and fractions from hog's stomach containing haemopoietin, the active substance effective in the treatment of pernicious anaemia.

2. An active extract has only been obtained by the method used by Buchner in the case of zymase. This involves the subjection of a mixture of fresh stomach tissue and sand to high pressure in a specially constructed apparatus.

3. Addition of alcohol to this press juice yields a precipitate in which the whole of the activity of the juice appears to be concentrated, the alcoholic filtrates being inactive.

4. Other active fractions have been prepared by fractional precipitation with alcohol and by fractional isoelectric precipitation.

5. Our experiments show that haemopoietin is more unstable than and has different properties from the active anti-anaemic principle in liver.

6. It is considered that the clinical experiments with gastric juice, the necessity for an extrinsic substrate, the association of pernicious anaemia with deficient enzyme secretion [Wilkinson, 1932, 2], the difficulty of extracting haemopoietin from stomach tissue, its digestion by the prolonged action of the proteolytic enzymes pepsin or trypsin, its association with the protein fraction of stomach press juice, its instability to heat and its sensitiveness to chemical treatment all harmonise with the view that haemopoietin is an organic substance of complex structure, probably a protein and possibly enzyme-like in its nature.

We have to thank the Honorary Physicians of the Manchester Royal Infirmary for placing at our disposal the patients necessary for these investigations and the Assistant Clinical Pathologists of the Manchester Royal Infirmary for their careful determinations of the blood counts. We also gratefully

acknowledge the invaluable help and facilities so freely given by Mr H. W. Baker, Dr K. Bullock and Mr J. Grier of the University of Manchester, and the generous assistance of Messrs Boots Pure Drug Co., Ltd., of Nottingham, who have made and supplied in quantity at our request various products and fractions examined.

Finally, we would thank the Medical Research Council, London, for a grant which has enabled one of us (L. K.) to take part in this work, which is being continued.

REFERENCES.

Albert and Buchner (1900). *Ber. deutsch. chem. Ges.* **33**, 266, 971.
Buchner (1897). *Ber. deutsch. chem. Ges.* **30**, 117, 1110.
—— (1898). *Ber. deutsch. chem. Ges.* **31**, 568.
Castle (1929). *Proc. Roy. Soc. Med.* **22**, 1214.
—— Heath and Strauss (1931). *Amer. J. Med. Sci.* **182**, 741.
—— —— —— and Townsend (1931). *J. Amer. Med. Assoc.* **97**, 905.
—— and Strauss (1932). *Lancet*, ii, 111.
—— Townsend and Heath (1930). *Amer. J. Med. Sci.* **180**, 305.
Cohn, Minot and co-workers (1927). *J. Biol. Chem.* **74**, Proc., lxix.
—— —— Alles and Salter (1928). *J. Biol. Chem.* **77**, 325.
—— McMeekin and Minot (1929). *Amer. J. Physiol.* **90**, 316.
—— —— —— (1930, 1). *J. Biol. Chem.* **87**, Proc., xlix.
—— —— —— (1930, 2). *Trans. Assoc. Amer. Physicians*, 45, 343.
Dakin, West and Howe (1930). *Proc. Soc. Exp. Biol. Med.* **28**, 2.
Fenger and Andrew (1927). *J. Biol. Chem.* **73**, 371.
Forbes (1927). *J. Biol. Chem.* **71**, 559.
Michaelis and Davidsohn (1910). *Biochem. Z.* **28**, 1.
Minot and Murphy (1926). *J. Amer. Med. Assoc.* **87**, 470.
Northrop (1930). *J. Gen. Physiol.* **13**, 739, 767.
Pekelharing and Ringer (1911). *Z. physiol. Chem.* **75**, 282.
Sherman and Schlesinger (1913). *J. Amer. Chem. Soc.* **35**, 1617.
West and Howe (1930). *J. Biol. Chem.* **88**, 427.
—— —— (1931). *J. Biol. Chem.* **94**, 611.
Wilkinson (1930). *Brit. Med. J.* i, 236.
—— (1931, 1). *Brit. Med. J.* i, 85.
—— (1931, 2). *Proc. Roy. Soc. Med.* **24**, 56.
—— (1931, 3). *Klin. Woch.* **10**, 791.
—— (1932, 1). *Brit. Med. J.* i, 325.
—— (1932, 2). *Quart. J. Med.* **1**, 361.
—— and Brockbank (1930). *Clin. J.* **59**, 475.
—— and Klein (1932). *Lancet*, i, 719.